

**PROCEEDINGS  
OF THE  
1st ANNUAL CONFERENCE  
ON  
ENVIRONMENTAL TOXICOLOGY**

**9-11 SEPTEMBER 1970**

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**AEROSPACE MEDICAL RESEARCH LABORATORY  
AEROSPACE MEDICAL DIVISION  
AIR FORCE SYSTEMS COMMAND  
WRIGHT-PATTERSON AIR FORCE BASE, OHIO**

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The voluntary informed consent of the subjects used in this research was obtained as required by Air Force Regulation 169-8.

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13. ABSTRACT
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This report is a compilation of the papers presented and the Proceedings of the 1st Annual Conference on Environmental Toxicology, sponsored by the SysteMed Corporation and held in Fairborn, Ohio on 9, 10, and 11 September 1970. Major technical areas discussed included toxicological evaluation of carbon monoxide, methodology, pathology, atmospheric contaminants, and toxicology of propellants and other military chemicals.

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## FOREWORD

The Conference on Environmental Toxicology was held in Fairborn, Ohio on 9, 10, and 11 September 1970. Sponsor was SysteMed Corporation under the terms of Contract F33615-70-C-1046 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Arrangements were made by the Toxic Hazards Research Unit of SysteMed Corporation, and the papers presented at this Conference by personnel of SysteMed Corporation represent research conducted under the cited contract. Dr. James D. MacEwen, Director, Environmental Sciences, SysteMed Corporation, served as Conference Chairman, and Mrs. Lois Doncaster, SysteMed Corporation, served as Conference Coordinator.

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PRINCIPAL SPEAKERS AND PARTICIPANTS

ADAMS, Jim D., Mr.  
Environmental Systems Branch  
USAF School of Aerospace Medicine  
Brooks Air Force Base, Texas

BACK, Kenneth C., Ph. D.  
Toxic Hazards Division  
Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

BEARD, Rodney R., M. D.  
Department of Community and  
Preventive Medicine  
Stanford University Medical Center  
Stanford, California

CARTER, Vernon L., Major, USAF, VC  
National Aeronautics & Space  
Administration  
Manned Spacecraft Center  
Houston, Texas

DAVIS, Harvey V., Ph. D.  
Toxic Hazards Research Unit  
SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

ERK, Stanley D., Mr.  
Toxic Hazards Research Unit  
SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

HALL, Arthur S., D. V. M.  
Department of Animal Science  
Oregon Regional Primate Research Center  
Beaverton, Oregon

HAUN, Charles C., Mr.  
Toxic Hazards Research Unit  
SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

HODGE, Harold C., Ph. D.  
Pharmacology Department  
University of California Medical Center  
San Francisco, California

HUETER, F. Gordon, Ph. D.  
Division of Health Effects Research  
DHEW, PHS  
Environmental Health Service  
Durham, North Carolina

INGRAM, Marylou B., M. D.  
Department of Radiation, Biology,  
and Biophysics  
University of Rochester Medical School  
Rochester, New York

JACOBSON, Keith H., Ph. D.  
Laboratory of Environmental Medicine  
Tulane University School of Medicine  
New Orleans, Louisiana

LEAHY, Harold F., Mr.  
Toxic Hazards Research Unit  
SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

LEON, Henry A., Ph. D.  
Biochemical Endocrinology Branch  
National Aeronautics & Space  
Administration  
Ames Research Center  
Moffett Field, California

MAC EWEN, James D., Ph. D.  
Toxic Hazards Research Unit  
SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

PRINCIPAL SPEAKERS AND PARTICIPANTS (CONT'D)

MAC KENZIE, William F., Major  
USAF, VC  
Toxic Hazards Division  
Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

O'DONNELL, Robert D., Captain,  
USAF, BSC  
Space Biology Laboratory  
Brain Research Institute  
UCLA Health Sciences Center  
Los Angeles, California

ROSE, Charles S., Lieutenant  
USNR, MSC  
U.S. Navy Toxicology Unit  
National Naval Medical Center  
Bethesda, Maryland

SALTZMAN, Bernard E., Ph.D.  
Research Professor of Environmental  
Health  
Kettering Laboratory  
University of Cincinnati  
Cincinnati, Ohio

SCHULTE, John, M.D.  
Department of Preventive Medicine  
The Ohio State University  
Columbus, Ohio

STEVENS, Robert K., Mr.  
Division of Chemistry and Physics  
National Air Pollution Control  
Administration  
Raleigh, North Carolina

STEWART, Richard D., M.D.  
The Medical College of Wisconsin  
Milwaukee, Wisconsin

THOMAS, Anthony A., M.D.  
Toxic Hazards Division  
Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

TISHER, C. Craig, M.D.  
Division of Nephrology  
Department of Medicine  
Duke University Medical Center  
Durham, North Carolina

TOLIVER, William H., Sr., Mr.  
Toxic Hazards Division  
Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

TOWNSEND, Frank M., M.D.  
Clinical Professor of Pathology  
University of Texas Medical School  
San Antonio, Texas

VERNOT, Edmond H., Mr.  
Toxic Hazards Research Unit  
SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

WANDS, Ralph C., Mr.  
Advisory Center on Toxicology  
National Academy of Sciences  
Washington, D.C.

WEINSTEIN, Ronald S., Major  
USAF, MC  
Toxic Hazards Division  
Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

WELCOMING REMARKS

James D. MacEwen, Ph. D.

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

Good morning, ladies and gentlemen, I'm your Chairman, Doug MacEwen. I'm your host and chairman for the Conference on Environmental Toxicology. I'd like to welcome you here to the Meeting on behalf of SysteMed Corporation and on behalf of the Aerospace Medical Research Laboratory here at Wright-Patterson Air Force Base.

The objective of this Conference is to promote a medium for the exchange for both ideas and experiences of people in the field of inhalation toxicology and the related sciences. This morning our first session will be on the toxicology of carbon monoxide. Before we get into this, I would like to mention that for those of you who have been here before, this is not the 6th Annual Conference on Atmospheric Contamination in Confined Spaces. We have changed the title because of the change in the objectives of the Air Force with the deemphasis of the Manned Orbital Laboratory program. The Conference this year is being sponsored directly by SysteMed Corporation, rather than by the Air Force, as it has been in the past. The 6570th Aerospace Medical Research Laboratory has had a change in command and this morning I would like to introduce to you the new Commander, Colonel Clinton Holt.

## INTRODUCTORY REMARKS

Clinton L. Holt, Colonel, USAF, MC

Commander  
Aerospace Medical Research Laboratory

On behalf of the Aerospace Medical Research Laboratory may I extend a warm personal welcome to the 1970 Conference on Environmental Toxicology. Since I have only recently assumed command of this Laboratory, I have not previously been personally familiar with this Conference but have heard many favorable comments about it and am familiar with its product. Having reviewed the proceedings of the recent conferences, I've been deeply impressed by the discussion and the quality of the papers presented. The matters to be addressed this year are certainly in keeping with an already established tradition of excellence and relevance. As we all know, environmental quality is a hot topic in many quarters and in its manifold aspects, for example socioeconomically, politically, culturally, and technologically. You who are assembled for this Conference undoubtedly have diverse interests in and impacts on one or more of these aspects. But I think it fair to assume that we do have one common reference, this is the application of technology to the solution of environmental quality problems. The material to be reported at this Conference represents basic and applied research of a broad ranging technical nature. I think it fair to assume that the technology to be reported here and advances therein may provide guidelines and stimulation to the development of procedures and applications that have wide applicability to the problem confronting us as a nation and, further than that, to assist us in the services to cope more effectively in the pursuit of our mission. My best wishes for a most rewarding scientific meeting.

SESSION I

TOXICOLOGY OF CARBON MONOXIDE

Chairman

Dr. Harold C. Hodge  
Pharmacology Department  
University of California Medical  
Center  
San Francisco, California

HEMATOLOGICAL EFFECTS OF LONG-TERM CONTINUOUS  
ANIMAL EXPOSURE TO CARBON MONOXIDE

Edmond H. Vernot

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

William F. MacKenzie, Major, USAF, VC

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

James D. MacEwen, Ph. D.

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

Paul N. Monteleone, M. D.

Johnstown Memorial Hospital  
Johnstown, Pennsylvania

Marilyn E. George

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

Paul M. Chikos, M. D.

14355 38th Street, N. E.  
Seattle, Washington

Kenneth C. Back, Ph. D.

Anthony A. Thomas, M. D.

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio  
and  
Charles C. Haun

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

## INTRODUCTION

It has long been acknowledged that polycythemia occurs as a consequence of chronic carbon monoxide (CO) exposure. The evidence concerning it and the course of its development has not, however, been completely unambiguous. Brieger (1944) exposed dogs to 100 ppm (115 mg/M<sup>3</sup>) of carbon monoxide for eleven weeks, 6 days a week, for an average of 5-3/4 hours a day. He found that hemoglobin (Hb) had reached maximum values in the period from 6 to 9 weeks, increasing from 13 to 15 grams percent. Similar increases were noted in red blood cell count. At the end of the exposure, however, values of both these parameters had returned to normal levels.

Killick (1948) exposed one human sporadically over a period of three years to concentrations of CO which produced moderate to high levels of carboxyhemoglobin (COHb) but she noted no increase in red blood cell count after acclimatization. Wilks et al (1959) exposed dogs 6-8 hours daily to 800 ppm (920 mg/M<sup>3</sup>) over a period of 36 weeks. Increases of hemoglobin and red blood cell counts occurred throughout the experiment, reaching 40 to 50% above normal values at termination. Wilks also determined the value of M in the Haldane Equation:

$$M = \frac{COHb}{O_2Hb} \frac{pO_2}{pCO}$$

He found a lower value, M = 200, for acclimatized dogs than for normal dogs, M = 235.

Very little work has been done on the effects of long-term continuous exposures to CO. This is true, notwithstanding the possibility that such experiments might have very interesting theoretical consequences arising from the fact that the test animals would not be subjected to cycles of exposure, and the physiological responses to continuous exposure might occur in a more orderly and analyzable fashion. The increasing interest in long-term habitation of closed systems such as spacecraft, nuclear submarines, and undersea laboratories provides a practical reason for long-term continuous studies since CO may be the most significant contaminant in such systems. This investigation was, therefore, undertaken in an effort to delineate the area of hematologic response to long-term continuous carbon monoxide exposure.

## METHODS AND MATERIALS

Usually, 4 Rhesus monkeys and 8 Beagle dogs comprised the experimental groups employed in this study. Exposures took place in specially designed chambers (McNerney and MacEwen, 1965) under simulated space cabin conditions, 260 Torr pressure and 68% oxygen, 32% nitrogen atmosphere. Entries into the chambers for feeding, cleaning, and sampling were made through airlocks so that no interruption of exposure occurred during these periods. Control groups of the same numbers of animals were housed in identical chambers under the same atmospheric conditions. Additional experiments were conducted in which groups of 12 Rhesus monkeys were exposed to 57.5 mg/M<sup>3</sup> CO for 100 day periods, again under space cabin conditions. CO concentrations were

monitored continuously by means of a commercial infrared analyzer (Beckman Instruments, Inc.). Table I summarizes the exposures and tests performed. One dog and two monkeys from the test group were held after conclusion of the 575 mg/M<sup>3</sup> experiment until hematological values returned to normal.

TABLE I  
SCHEDULE OF LONG-TERM CARBON MONOXIDE EXPOSURES

CO Concentration mg/M <sup>3</sup>	Experimental Animals, No.	Length of Exposure	Tests Performed	Sampling Frequency
57.5	Rhesus Monkeys, 12	100 days	Hb HCT RBC	monthly monthly monthly
115	Rhesus Monkeys, 4 Beagle Dogs, 8	26 weeks	COHb Hb HCT RBC	biweekly biweekly biweekly biweekly
230	Rhesus Monkeys, 12	100 days	COHb Hb HCT RBC	monthly monthly monthly monthly
460	Rhesus Monkeys, 4 Beagle Dogs, 8	11 weeks	COHb Hb HCT RBC	biweekly biweekly biweekly biweekly
575	Rhesus Monkeys, 4 Beagle Dogs, 8	13 weeks	COHb Hb HCT RBC	biweekly biweekly biweekly biweekly
	Beagle Dogs, 2		Blood Viscosity	monthly
	Rhesus Monkeys, 2		Blood O <sub>2</sub> Content	monthly
	Beagle Dogs, 2		Blood pO <sub>2</sub>	monthly
	Rhesus Monkeys, 4 Beagle Dogs, 4		Blood Volume	termination

Hematological parameters were determined employing blood drawn from the jugular vein and using standard clinical laboratory procedures. Hemoglobin was determined by the cyanmethemoglobin method, hematocrit was measured using microhematocrit tubes, and red blood cells were counted by a commercial instrument (Coulter Electronics, Inc.). Reticulocytes were counted in 1000 red cells after staining with brilliant cresyl blue. Blood COHb was measured by the gas chromatographic method of Goldbaum, Schloegel and Dominguez (1963).

The same groups of animals were exposed to 460 mg/M<sup>3</sup> and 575 mg/M<sup>3</sup> CO. Initially, it was expected that severe toxic signs might be evidenced on long-term exposures to 460 mg/M<sup>3</sup>. When, after 10 weeks, it was apparent that this was not the case, the concentration was increased to 575 mg/M<sup>3</sup> for the rest of the experimental period, 13 weeks.

The exposures of Rhesus monkeys to 57.5 mg/M<sup>3</sup> and 230 mg/M<sup>3</sup> were conceived primarily for testing behavioral responses. The nature of these experiments precluded using control animals since the behavioral responses of the monkeys were compared to their own preexposure patterns. Therefore, hematologic changes in these animals were compared to preexposure baseline values rather than to concurrent controls.

A number of additional tests were performed on the blood of selected animals exposed to 575 mg/M<sup>3</sup>. Blood volume measurements utilized the method of Logsdon (1968) employing <sup>51</sup>Cr for total blood volume and <sup>59</sup>Fe for plasma volume. Blood viscosities were determined following the technique of Rand et al (1964). Arterial blood gas determinations were made on blood sampled by femoral artery cutdown after anesthetizing the animals with 30 mg/kg pentobarbital. Oxygen content and saturation were determined using Van Slyke's standard manometric technique. Potentiometric pO<sub>2</sub> measurements were made using a commercial instrument (Instrumentation Laboratory, Inc.).

In addition to the above tests, standard techniques were utilized to measure white blood cell count, osmotic fragility, and coagulation parameters. None of these tests showed any differences between exposed and control animals.

Two monkeys and one dog were retained for postexposure weekly blood samplings until their hematology values became normal once again. For both species this occurred in six weeks.

## RESULTS AND DISCUSSION

In figure 1 are plotted the biweekly means of RBC, Hb and HCT of dogs exposed to 460 mg/M<sup>3</sup> and subsequently, to 575 mg/M<sup>3</sup> of CO. All parameters rose significantly by the end of the second week of exposure to 460 mg/M<sup>3</sup> and reached equilibrium by the end of the fourth week. Upon further increase in CO concentration to 575 mg/M<sup>3</sup> at the tenth week, the hematological values again rose, reaching a new equilibrium in two to four weeks. The mean relative increases over controls at 460 mg/M<sup>3</sup>, RBC-33%, Hb-30%, HCT-27% and at 575 mg/M<sup>3</sup>, RBC-44%, Hb-41%, HCT-38%, indicate that the poly-

cythemia was normocytic and normochromic. In contrast to the results obtained by Brieger (1944) in his interrupted exposures, there were no decreases in hematological values upon prolonged exposure to moderate CO concentrations.

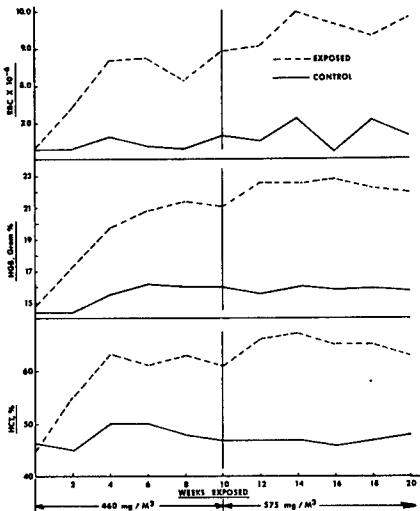


Figure 1. EFFECT OF CO EXPOSURE ON HEMATOLOGY - DOGS

Figure 2 is a graph of the blood parameters of the monkeys in the same exposure. An obvious difference from dogs is that no significant increase in any of the variables occurred after two weeks of exposure. Thereafter, increase was rapid, and equilibrium was established at the end of the fourth week of exposure. As in dogs, further rises in all parameters were noted after the CO concentration was increased from 460 to 575 mg/M<sup>3</sup>. The relative increases of all parameters at 460 mg/M<sup>3</sup>, RBC-39%, Hb-40%, HCT-38%, and 575 mg/M<sup>3</sup>, RBC-60%, Hb-58% and HCT-59%, were greater than for dogs, although the absolute values never quite attained those reached by dogs. For whatever significance it may have, the monkeys in this experiment began the exposure with about three grams/percent less hemoglobin than the dogs. This may explain why monkeys have a greater increase in hematological values than dogs in response to CO intoxication.

As in the case of the dog, consideration of the increases in RBC, Hb and HCT indicates that the blood indices were unchanged and that the polycythemia was normocytic and normochromic.

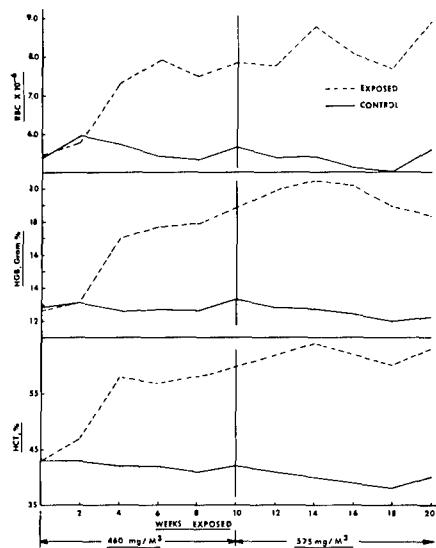


Figure 2. EFFECT OF CO EXPOSURE ON HEMATOLOGY - MONKEYS

The exposure of animals to lower CO concentrations resulted in the same type of hematological response. The values increased over the first month of exposure and stabilized at some elevated point for the rest of the exposure. The differences between equilibrium exposure values of hemoglobin and baseline or control values are plotted against chamber CO concentrations in figure 3. Hematocrit differences are shown in figure 4. The lines were fitted using standard regression techniques. Analysis of the data gave no indication that non-linear curves would provide more satisfactory fits, and the 95% confidence limits of the slopes were such that all lines probably passed through the origin. The effects on hematocrit and hemoglobin appear, therefore, to be linear over the whole range of carbon monoxide concentrations studied, and identical increases occur whether the carbon monoxide concentration goes from zero to 115 mg/M<sup>3</sup> or from 460 to 575 mg/M<sup>3</sup>. The other inference to be drawn from figures 3 and 4 is the probability that exposure to any level of carbon monoxide will lead to a real increase in the hematological parameters.

Both dogs and monkeys showed slight reticulocyte increases over controls from 2 to 8 weeks into the 460 mg/M<sup>3</sup> exposure. Values then returned to normal to become marginally elevated for two weeks when the CO concentration is raised to 575 mg/M<sup>3</sup>. Little significance can be attached to these increases because of the inherent imprecision of the technique.

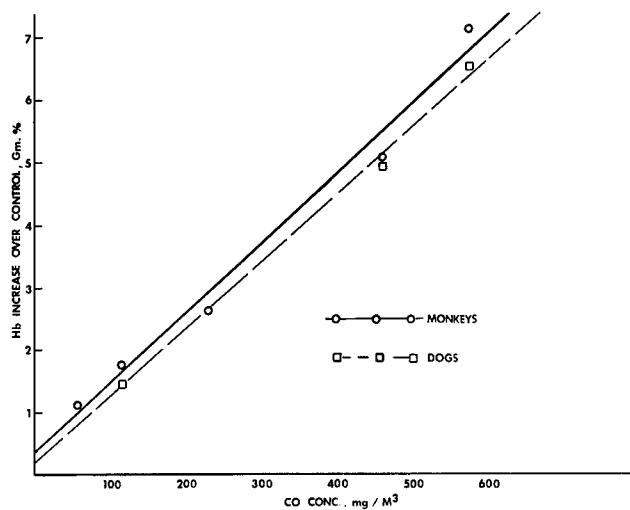


Figure 3. HEMOGLOBIN INCREASES AFTER CO ACCLIMATIZATION

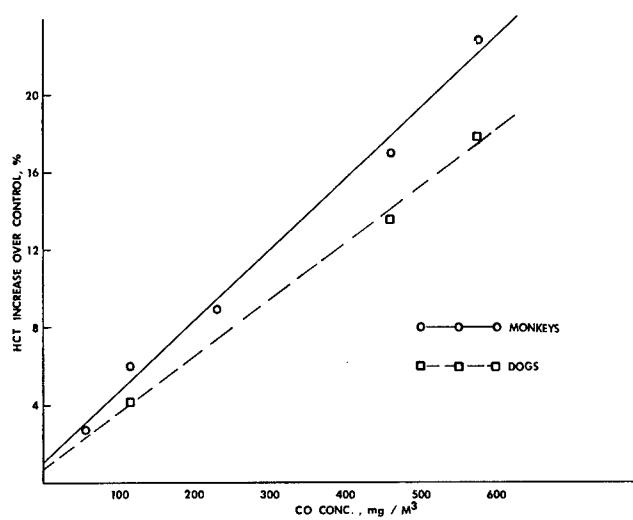


Figure 4. HEMATOCRIT INCREASES AFTER CO ACCLIMATIZATION

TABLE II  
MEAN EQUILIBRIUM COHb CONCENTRATIONS OF BLOOD  
OF ANIMALS EXPOSED TO CO

<u>CO Conc.</u>	<u>COHb</u>	
<u>mg/M<sup>3</sup></u>	<u>% Saturation</u>	
	<u>Rhesus Monkeys</u>	<u>Beagle Dogs</u>
115	12.0	12.8
230	21.3	--
460	31.6	33.2
575	38.5	39.5

Table II lists the equilibrium concentrations of carboxyhemoglobin in the blood of monkeys and dogs exposed to various concentrations of carbon monoxide. If following Killick (1948) the assumption is made that the concentration of reduced hemoglobin in arterial blood is negligible, then  $\% \text{O}_2\text{Hb} = 100 - \% \text{COHb}$  and the ratio  $\frac{\% \text{COHb}}{100 - \% \text{COHb}}$  plotted against chamber carbon monoxide concentrations should yield straight lines for each species. Figure 5 demonstrates that this is indeed the case for monkeys and dogs, and extrapolation of the lines to the point where blood is 50% saturated with carboxyhemoglobin should allow us to calculate the M constant as follows:

$$M = \frac{\text{COHb}}{\text{O}_2\text{Hb}} \times \frac{\text{pO}_2}{\text{pCO}}$$

where  $\text{pO}_2$  and  $\text{pCO}$  are arterial partial pressures. If  $\text{COHb} = 50\% = \text{O}_2\text{Hb}$ ,  $M = \frac{\text{pO}_2}{\text{pCO}}$  at 50% saturation. Making the further assumption that alveolar partial pressures may be substituted for arterial values;  $\text{pO}_2$  in mm = 110

$$\text{pCO in mm} = \text{chamber conc. (mg/M}^3\text{)} \times 6.63 \times 10^{-4} \times \frac{223}{260}$$

$$\begin{aligned} M (\text{dogs}) &= 218 \\ M (\text{monkeys}) &= 207 \end{aligned}$$

The calculation for  $\text{pCO}$  converts chamber CO concentration to mm and corrects for the dilution caused by water vapor saturation of inspired atmosphere. The M value for dogs compares well with  $M = 220$ , determined *in vitro* by Allen and Root (1957) at arterial pH 7.4. Furthermore, since the constants were calculated from data obtained on long-term carbon monoxide exposures, it appears that the length of exposure has little or no effect on the equilibrium between oxy- and carboxyhemoglobin. A search of the literature indicated that this is the first determination of the M constant for monkeys.

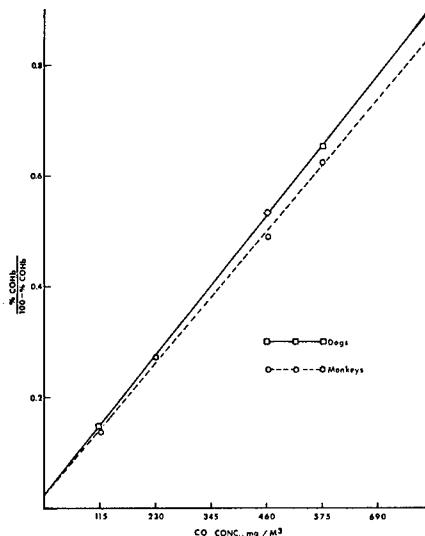


Figure 5. LINEARITY OF RATIO  $\frac{\% \text{ COHb}}{100 - \% \text{ COHb}}$  WITH CO CONCENTRATION

Some investigators (Lilienthal et al, 1946; Wilks et al, 1959) have determined arterial  $pO_2$  by physical means for calculation of M. They used alveolar pCO, primarily because there are no methods for measuring arterial pCO. It appears however that, if there is an alveolar-arterial  $pO_2$  difference this must result in an alveolar-arterial pCO difference since:

$$pCO = \frac{COHb}{O_2Hb} \times \frac{pO_2}{M}$$

and since arterial  $\frac{COHb}{O_2Hb}$  is constant under fixed atmospheric ( $pO_2$ ) and contaminant con-

ditions. Therefore, it can be seen that arterial pCO is a linear function of arterial  $pO_2$ , and using measured arterial  $pO_2$  and the calculated alveolar pCO to calculate M may result in greater error than when alveolar partial pressures are used for both.

With the Haldane constants determined, we were able to construct theoretical oxygen dissociation curves for the blood of monkeys and dogs exposed to  $575 \text{ mg/M}^3$  CO using calculations developed by Roughton and Darling (1944). Figure 6 compares a normal dog oxygen dissociation curve (solid) calculated from the data of Bartels and Harms (1959) for blood containing 15.8 g% hemoglobin with curves calculated for blood from animals exposed to  $575 \text{ mg/M}^3$  CO at 260 Torr, 68% oxygen, long enough to attain the equilibrium concentration of COHb 39.5%. The dashed curve represents blood which has acclimatized to the exposure by increasing its hemoglobin to 22.3 g%. The dotted curve represents blood from a short exposure to which the animal has not yet started to acclimatize by increasing its hemoglobin. Even in the acclimatized animal, the arterial

blood does not carry as much oxygen as normal, suffering a loss of 14% at nominal alveolar  $O_2$  partial pressures of 110 Torr. As expected, the dissociation curve is shifted to the left, signifying that oxygen is delivered to the tissues at lower partial pressures than normal. Without any compensatory increase in total hemoglobin, equilibrated blood shows a 50% drop in saturated oxygen content. This curve, too, is shifted to the left but not as significantly as in compensated blood.

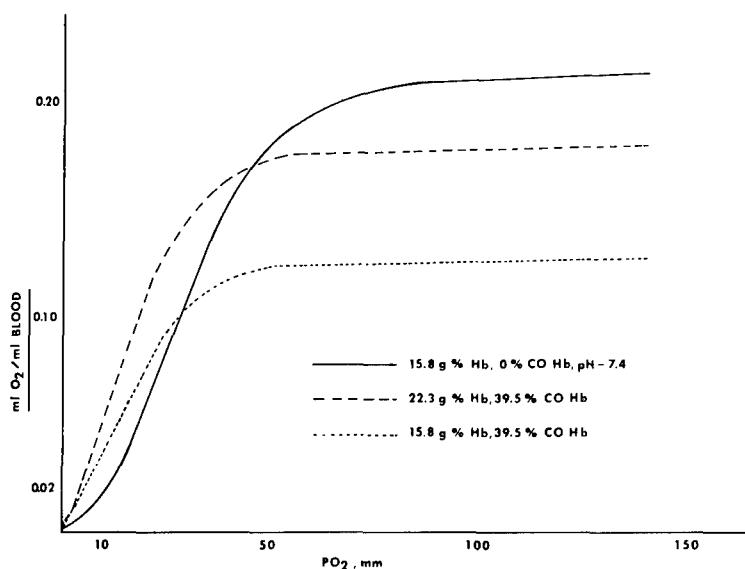


Figure 6.  $O_2$  DISSOCIATION CURVES - DOGS

A similar series of theoretical dissociation curves for monkeys is shown in figure 7. Here the normal curve was calculated for blood containing 12.5 g% total hemoglobin using Parer's (1967) data. The exposed blood is 38.5% saturated with carbon monoxide and the compensated exposed blood has increased its hemoglobin to 19.5 g%.

As far as total oxygen capacity is concerned, the compensated monkey blood is very near to normal, carrying 55% more oxygen than the uncompensated blood. However, the pronounced left shift of the curve demonstrates that this oxygen is delivered at lower partial pressures.

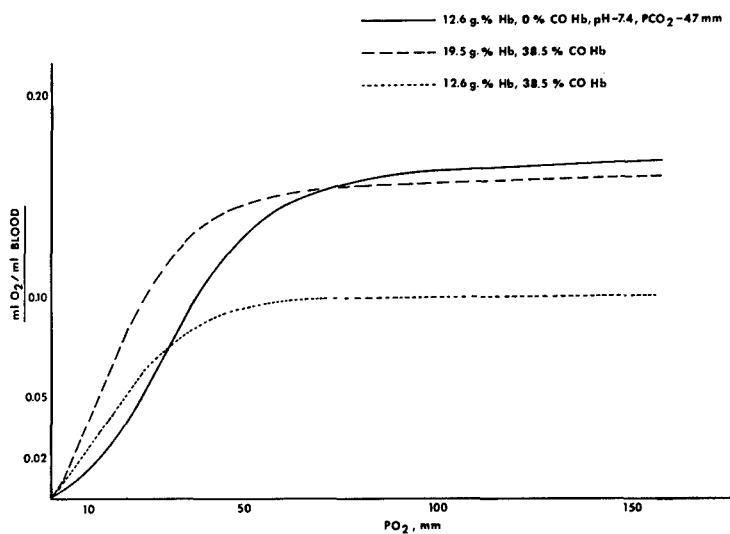


Figure 7.  $O_2$  DISSOCIATION CURVES - MONKEYS

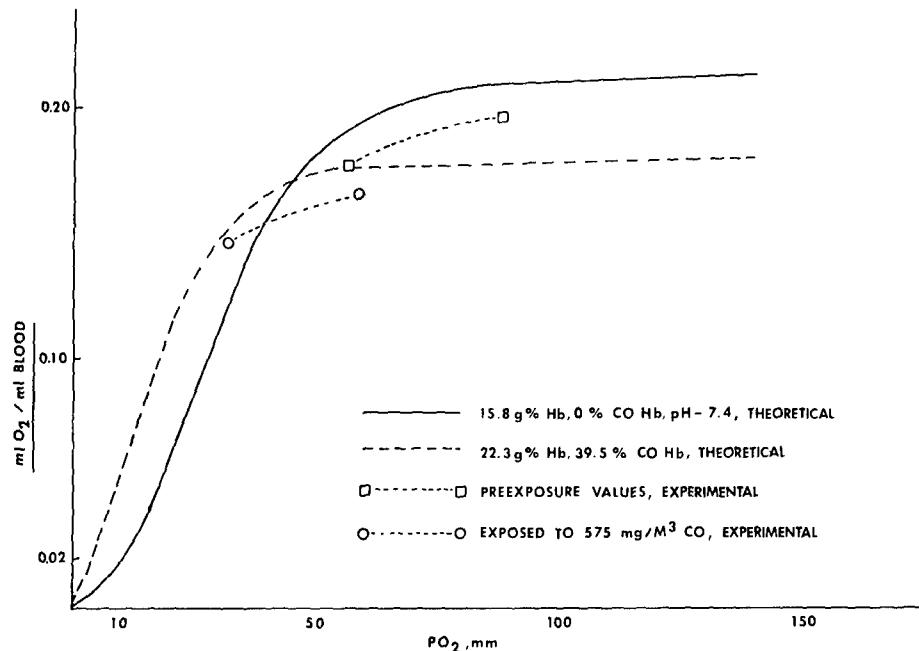
The dissociation curves for carboxyhemoglobin containing blood shown in figures 6 and 7 are plotted under the assumption that no other factors are present which might tend to shift the curve to the right or further to the left. Recent investigators (Benesch and Benesch, 1967; Brewer et al, 1970; Eaton and Brewer, 1968) have shown that blood does contain substances such as ATP or 2, 3-diphosphoglycerate which (at least in vitro) tend to shift the oxygen dissociation curves to the right. It has also been demonstrated that under certain conditions the concentration of 2, 3-DPG in blood can increase significantly. If this had occurred in our animals, then the dissociation curves would not present a valid picture of oxygen transport. Although ATP and 2, 3-DPG were not measured in this study, determinations of arterial and venous oxygen content and partial pressure made preexposure and after equilibration to 575 mg/M<sup>3</sup> CO in dogs demonstrated that there were no phosphate induced shifts. Table III presents the data obtained.

There are three observations to be made concerning the data in table III.

1. The oxygen content is lower in arterial blood from exposed animals, reflecting the decreased oxygen carrying capacity.
2. The arterial  $pO_2$  is lower in exposed animals, a phenomenon which has frequently been noted as a result of carbon monoxide intoxication. Filley (1970) has presented an explanation based upon the changes in the oxygen dissociation curve caused by the presence of carboxyhemoglobin.
3. The differences between oxygen content of arterial and venous blood are identical in baseline and exposed animals. Although the A-V difference in blood oxygen content is not an absolute measure of tissue oxygenation, this does indicate that, over the locality supplied by the sampled blood, the same volume of oxygen was transferred in test and control animals.

TABLE III  
BLOOD GAS VALUES, DOGS

		ml O <sub>2</sub> /ml Blood	Arterio-Venous O <sub>2</sub> Difference,	
	% Saturation	14.8 gm% Hb	ml O <sub>2</sub> /ml Blood	pO <sub>2</sub> , mm
<u>Preexposure</u>				
Arterial	90.9	0.196		87.8
Venous	81.8	0.176	0.020	56.5
<u>Exposed, 575 mg/M<sup>3</sup> CO</u>				
Arterial	54.8	0.166		58.6
Venous	48.0	0.146	0.020	31.9

Figure 8. BLOOD O<sub>2</sub> VALUES - DOGS

In figure 8, the experimental data shown in table III are plotted and compared with the theoretical oxygen dissociation curves for unexposed and acclimatized exposed animals. The experimentally determined blood oxygen values correspond very well to the theoretical plots, all points being approximately 0.01 ml of oxygen below the calculated curve. From this, one can draw the inference that, no matter what concentrations of ATP or 2, 3-DPG are present, no right shift of the oxygen dissociation curve has occurred, and the only factor operating on it is the presence of carboxyhemoglobin.

As has been noted, measurement of blood indices demonstrated that exposure caused no significant change in individual erythrocyte parameters. Total blood volume increased in both animal species while plasma volume remained constant as shown in table IV, demonstrating that the volume of the circulatory system had increased to accommodate the increased hematocrit. These measurements were made at exposure termination and provide no information concerning the rate of this accommodation.

TABLE IV  
BLOOD VOLUMES AFTER EXPOSURE TO 575 mg/M<sup>3</sup> CO

Total Blood Volume, ml/kg		Plasma Volume, ml/kg	
Control	Exposed	Control	Exposed
Rhesus Monkeys			
72	100	50	47
Beagle Dogs			
100	131	58	60

It was to be expected that development of a pronounced polycythemia would have profound effects on the physical characteristics of the blood, in particular blood viscosity. A graphic exposition of this effect is presented in figure 9 which plots the viscosity of dog whole blood and plasma samples measured at 46 sec<sup>-1</sup> shear rate as a function of hematocrit. The plotted points represent mean values measured on blood sampled from two control dogs and two dogs acclimatized to 575 mg/M<sup>3</sup> CO. Measurements were taken at other shear rates and showed the expected non-Newtonian behavior of whole blood, i. e., increasing viscosity with decreasing shear rate. Plasma demonstrated Newtonian behavior, giving constant viscosity at all shear rates tested.

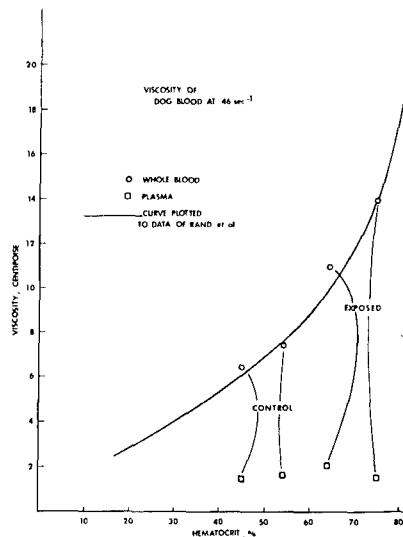


Figure 9. VISCOSITY OF DOG BLOOD - CONTROLS AND ACCLIMATIZED TO 575 mg/M<sup>3</sup> CO

Figure 9 illustrates the fact that the viscosity of the polycythemic blood is a function only of the increased hematocrit. The curve in figure 9 is fitted to the data of Rand et al (1964) taken at a shear rate and temperature comparable to this study and using reconstituted human blood with hematocrits of from 20% to 80%. All the viscosities of the blood of control and exposed dogs shown as individual points in the figure lie very close to this line and all of the plasma samples have essentially the same viscosity, identical to that found by Rand et al for human plasma, 1.5 centipoise. It is seen that the blood with a 75% hematocrit lies on a point of the curve where the viscosity is increasing very rapidly, and further small increases in hematocrit would lead to large viscosity increases. The evidence previously presented that hematocrit is a linear function of CO concentration implies that further small increases in atmospheric CO concentration beyond 575 mg/M<sup>3</sup> would produce large viscosity increases resulting in significant effects on blood circulation.

#### SUMMARY

Long-term continuous exposures of dogs and monkeys to CO produce a series of measurable changes resulting from the development of normocytic, normochromic polycythemia as shown below:

1. Increase in hematological parameters appears to be a linear function of carbon monoxide concentration from the lowest, 57.5 mg/M<sup>3</sup>, to the highest, 575 mg/M<sup>3</sup>, tested.

2. The monkeys responded relatively more profoundly than dogs by increases in hematological values.
3. The Haldane M constant applies to the in vivo formation of COHb, and can, in fact, be calculated using inhalation data.
4. The effect of exposure to high CO concentration on the  $O_2$  dissociation curve appears to be explainable on the basis of Roughton's assumptions alone, and the curve does not show any right shift attributable to ATP or 2, 3-DPG.
5. The polycythemia developed as a result of chronic exposure to high concentrations of CO does not compensate completely in blood  $O_2$  transport.
6. Under the conditions of this study, arterio-venous difference in oxygen content provides no evidence of tissue hypoxia in equilibrated dogs.
7. The increase in hematocrit in acclimatized dogs and monkeys is matched by an equal increase in total blood volume.
8. The viscosity of polycythemic blood can be explained on the basis of HCT increases alone.

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PATHOLOGY IN ANIMALS EXPOSED TO HIGH CONCENTRATIONS  
OF CARBON MONOXIDE FOR SIX MONTHS

William F. MacKenzie, Major, USAF, VC

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

Roman L. Patrick, Jr., M.D.

Laboratory for Experimental Biology  
St. Louis, Missouri  
and  
Paul N. Monteleone, Jr., M.D.

Johnstown Memorial Hospital  
Johnstown, Pennsylvania

This paper is concerned with the anatomic pathology found in several species of animals due to long term continuous exposure to high levels of carbon monoxide (CO); however, because of the uniqueness of this experiment a brief description of the clinical appearance of the animals is included.

METHODS

The details of the exposure were presented in the paper given by Mr. Verno and will only be summarized here. Sixty-eight rats, 40 mice, 2 baboons (*Papio* species), 4 rhesus monkeys (*M. mulatta*), and beagle dogs were exposed to 460 mgm/M<sup>3</sup> CO for 71 days followed by 575 mgm/M<sup>3</sup> CO for 97 days at 5 psi pressure and a 68% O<sub>2</sub>, 32% N<sub>2</sub> atmosphere. One baboon and a similar number of other animals were kept as controls under identical conditions with the exception of the CO. The animals were under periodic observation by technicians who became completely familiar with the animals and could recognize subtle changes in activity. The same crew drew blood on a bi-weekly basis and these men were questioned as to any changes in the ability of the animals to resist venipuncture. At the end of the experiment, simple neurologic examinations were performed on the dogs consisting of foot placing, toe-pinch, hopping and righting reflexes. Retinal reflexes were tested on all large animals.

One exposed and two control dogs and two exposed and two unexposed monkeys were held four months for reversibility studies. All others were necropsied within four hours of the termination of the experiment. All animals received a detailed gross examination. Organ weights were obtained on the rats and dogs, organ/body weight ratios calculated, and statistical comparisons performed. In the dogs, measurements were taken of the ventricular thickness, septal thickness, circumference and length. Food was withheld from all animals for 12 hours before necropsy. Two different techniques were used in the dogs and primates. Two dogs from each group and the baboons were anesthetized with pentobarbital and perfused with 10% neutral buffered formalin simultaneously via the abdominal aorta and both carotid arteries at 120 mm Hg pressure. The lungs were expanded in situ with neutral buffered formalin at 20 cm of water pressure. Sections of the brain, heart, lungs, liver and kidneys were obtained for embedding in plastic. An eye was removed and placed immediately in formalin after which representative sections of all organs were taken. The other large animals were anesthetized and exsanguinated via the femoral arteries. An eye was immediately removed and put into fixative; the lungs were weighed and then expanded with formalin. The rats were anesthetized and exsanguinated in the same manner. The mice were killed by breaking their necks.

Light microscopic examination was performed on tissues from all large animals and on 8 rats and 8 mice from exposed and control groups. In the large animals, tissues examined were sections of heart to show right and left atria, right and left ventricle; high intraventricular septum including a portion of right atrium and coronary arteries, papillary muscles and ascending aorta; sections of the brain to show anterior and posterior basal ganglia, cerebrum, hippocampus, subcortical white matter, cerebral peduncles, mid-brain, substantia nigra, medulla and cerebellum at the level of pons, and the posterior medulla; multiple sections of lung; stomach and intestine; reticuloendothelial system, kidney, urinary vesicle, liver and biliary system, pituitary, adrenal, thyroid, gonads, coronal section of eye, and any other tissue showing gross abnormality. Frozen sections of heart stained with oil red O were prepared. Light microscopy of paraffin embedded material was performed at the Laboratory for Experimental Biology, St. Louis, Missouri, with the exception of frozen sections, sections of the eye, and tissues from those animals held for hematologic studies at the end of the experiment; these were performed at the Aerospace Medical Research Laboratory. Sections of lung, heart, liver and kidney embedded in epon 812 were cut at one micron, stained with methylene blue and studied by oil emersion light microscopy. Other materials were processed and stained in standard ways. Special stains were used as necessary.

## RESULTS AND DISCUSSION

The immediate response to exposure of all species was depression of activity. This was much more evident in the large animals. The primates were very quiet and sat hunched in their cages. The usually excited beagles were lethargic and morose. Food consumption dropped and we thought we might lose some animals. In three days there was an obvious improvement with no animal losses. Within a week they appeared re-

latively normal and in two weeks all responded to the first blood sampling with a vigor indistinguishable from the control animals. For the rest of the exposure period there was no clinical evidence of deleterious effect based on observed behavior as shown by simple neurologic examination, retinal reflexes, and ability to struggle when restrained for blood sampling. There was a reddening of unpigmented areas of the skin. During the exposure there was no extraordinary number of deaths. Of the exposed animals, deaths were limited to rats-- five of the 68 exposed died. In the control group one mouse, one monkey and two rats died. The diagnosis for the controls were: Monkey - gastric tympany; mouse - lymphosarcoma; in the rats, postmortem autolysis was too advanced for diagnosis. This occurred because entry into the Thomas Domes is usually not made to recover dead rodents because of extensive safety procedures. Four of the exposed rats died in the last one-third of the experiment, three in the last 20 days. Only in the last two deaths were the rats preserved well enough for diagnostic procedures. This number of deaths is not considered excessive.

The changes noted in the exposed animals at necropsy reflected the hematologic changes reported in Mr. Vernot's paper. All organs gave the impression of being engorged with blood and the hearts of the dogs, rats and mice appeared enlarged. However, comparison of heart measurements between exposed and control dogs showed no significant difference with a T test. No other organ weight differences were noted in the dogs; however, in the rats the mean weights for the kidneys and livers were lighter in the exposed animals and the mean weights of the hearts and spleens were heavier. Since the exposed animals were lighter than the controls, this could explain the difference in kidney and liver weight. This difference in body weights between the two groups was not statistically significant. The increases in heart and spleen weight were also found in organ/body weight ratios and were statistically significant. Considering the increase in hematocrit and blood viscosity, this increase in heart and spleen weight was not surprising. Subjectively, from gross examinations, the increase in heart weight seemed to be predominately in the left ventricular musculature.

There were no microscopic lesions found in any species that could be associated with the exposure with one possible exception. The two rats that died on the 166th day of exposure had lesions that could have been complicated by circulatory failure induced by excessive blood volume and viscosity. At necropsy both of these animals had similar lesions consisting of congestion of all organs, hemorrhage of the small intestine and pulmonary edema. Microscopic examination confirmed the severe pulmonary congestion and edema. The hemorrhage of the small intestine occurred not only within the lumen of the intestine but also within the mesenteries and submucosa. Sections of the heart showed large wide muscle fibers, congested vasculature, and an occasional deeply stained muscle fiber. This is a non-specific change in the heart and can occur in healthy animals (Selye et al, 1968). The diagnosis of these two animals is not certain but it is possible that circulatory failure contributed to their deaths. None of the other rats exposed to CO showed any indication of impending circulatory collapse.

Although no lesions associated with the exposure were found in the dogs, two interesting diseases unassociated with the exposure were found in both exposed and

control animals. The first caused small granulomata within the lung occasionally containing the debris of a dead nematode larva and also areas of chronic peribronchitis and areas of chronic interstitial pneumonitis. Fortunately, the etiologic agent was found in examining the last of the dogs studied. This was Filaroides milksi. The second disease was a severe orchitis in a control dog typical of that caused by Brucella canis. Brucellosis is known to have occurred in the kennel producing the dog.

Lewey and Drabkin described profound CNS lesions in dogs exposed to 0.01 vol % CO five and a half hours a day, six days a week, for 11 weeks. Ehrich et al (1944) described severe cardiac changes in dogs from the same study. Lindenberge et al (1962) also described CNS and heart changes at 58 and 155 mgm/M<sup>3</sup>. Although our exposure was both higher and longer, no lesions in these organs could be demonstrated. There have been other studies where long term exposure of animals to CO has yielded essentially negative results; however, these studies often get short shrift in reviews of the literature. Van Bogaert et al in 1938 attempted to keep rhesus monkeys at 30% HbCO for 6 months but actually varied from a low of 7.5% to a high of 60% HbCO. Upon detailed examinations of the CNS, no lesions could be found. Musselman et al (1959) exposed rats, rabbits, and dogs for three months at 58 mgm/M<sup>3</sup> with no effects. Clark and Otis (1952) exposed mice to increasing concentrations until they acclimated to 0.12% after 14 days and subsequently exposed them to high altitude hypoxia. They reversed the process, exposing mice to reduced atmospheric pressure followed by exposure to CO at 0.25% and found that the two stresses were similar and that each provided increased tolerance to the other.

Astrup et al (1967) reported that high CO caused intimal changes in the aortas of rabbits and increased lipid deposition in the aorta when the rabbits were on high cholesterol diets. Our study did not find any intimal changes in the aortas of the species we studied. Our animals were on commercial laboratory diets rather than "high cholesterol" diets; however, no increased lipid deposition could be demonstrated in the species we used.

## SUMMARY

Rats, mice, baboons, rhesus monkeys, and beagle dogs were exposed to 460 mgm/M<sup>3</sup> CO for 71 days followed by 575 mgm/M<sup>3</sup> CO for 97 days in a 68% O<sub>2</sub>, 32% N<sub>2</sub>, 5 psi environment. Anatomic changes found were confined to rodents and consisted of an increase in heart and spleen weights. This can be explained on the basis of increased RBC volume and blood viscosity. The possibility that the rats had begun to reach the maximum tolerable compensatory increase in RBC volume must be considered based on the death of two rats on the 166th day of exposure with lesions suggestive of circulatory complications. No anatomic changes were found in the other species. Based on the pathologic studies, it is indicated that CO has no direct effect on the body that produces a degenerative anatomic change. The animals at risk in this experiment were young healthy adults in the case of the rodents and dogs and healthy adolescent primates. They are not representative of the human population at risk in a civilian community or, for that matter, in the Air Force as a whole. However, it does seem that the body's ability to adjust to high CO levels is much greater than had been previously suspected and is limited mainly by available circulatory reserve.

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## DISCUSSION

DR. HODGE (University of California Medical Center): We have time for a question or two. Would someone like to ask Major MacKenzie a question?

DR. INGRAM (University of Rochester Medical School): Did you have an opportunity to look at the bone marrow?

MAJOR MAC KENZIE (Aerospace Medical Research Laboratory): We did bone marrow slides--for some morphology and ME ratios.

DR. INGRAM: But not tissue sections?

MAJOR MAC KENZIE: Yes, we did and they were normal. No, I cannot tell you on hyperplasia of red marrow, we didn't do them.

DR. HODGE: Dr. Ingram just half completed her question and Major MacKenzie answered it. I'd like to know what the rest of the question is.

MAJOR MAC KENZIE: Oh, I'm sorry.

DR. INGRAM: I was just wondering particularly whether you made any estimates of how much of the marrow was red and whether there was any extramedullary hematopoiesis in the dogs?

MAJOR MAC KENZIE: No, there was no extramedullary hematopoiesis. There was no noticeable increase in extramedullary or splenic hematopoiesis in the rodents; however, the spleens were larger. We did not do long sections of the long bones trying to estimate any hyperplasia; unfortunately the bones went another direction before we did this.

DR. TISHER (Duke University Medical Center): I was just wondering if you had made any ultrastructural observations? You did indicate that the tissue was taken for electron microscopy.

MAJOR MAC KENZIE: No, we haven't. We have made thick sections or thin sections in speaking of E.M., on these blocks and have viewed them under oil immersion light microscopy. There are no noticeable changes whatsoever. There are some interesting differences due to species. For instance, the baboons have quite a lot of wear and tear pigment in their hearts, even though they are rather young, a few things like this, but no differences between tissues from exposed and controls.

DR. HODGE: May I ask out of ignorance, are there changes that have been described in electron micrographs ascribed to carbon monoxide?

MAJOR MAC KENZIE: No, but we hope to do this. There have been changes described in the epithelium of the lung, however. On the second study done more carefully by splitting the lung into two sections, exposing one to carbon monoxide and the other to oxygen, there were no changes. There have been none at all. There have been changes described in heart muscle, particularly in mitochondria at high altitude.

HUMAN SLEEP PATTERNS AND PSYCHOMOTOR PERFORMANCE DURING  
EXPOSURE TO MODERATE CONCENTRATIONS OF CARBON MONOXIDE

Robert D. O'Donnell, Captain, USAF

UCLA Health Sciences Center  
Los Angeles, California

Paul M. Chikos, M. D.

14355 38th Street, N. E.  
Seattle, Washington  
and  
James Theodore, M. D.

Stanford University Medical Center  
Stanford, California

INTRODUCTION

Carbon monoxide (CO) effects are generally believed to be mediated through tissue hypoxia (Dinman, 1968). Since the central nervous system is extremely sensitive to oxygen deprivation, it would be expected that even low level exposures to CO would produce observable changes in human performance. Yet experimentation in this area has not produced as clear a picture as one would have expected.

It is accepted that subjective symptoms of CO exposure rarely occur below carboxyhemoglobin (COHb) levels of 20 percent, while most acute signs of cardiovascular, respiratory, and central nervous system embarrassment occur at COHb levels greater than 30 percent (Haldane, 1927). However, a number of investigators have indicated that human performance is impaired at COHb levels as low as 2 to 5 percent. Visual discrimination was measured by MacFarland, Roughton, Halperin, and Niven (1944) who demonstrated impairment with COHb levels of 4 percent. Similarly, Lilienthal and Fugitt (1946) reported impaired flicker fusion at 6,000 feet with COHb levels of 5 to 10 percent. However, another study (Vollmer, King, Birren and Fisher, 1946) found no changes in flicker fusion, visual perimetry, or ataxia as a function of COHb level up to 22 percent at simulated altitudes of 10 and 15 thousand feet.

With respect to cognitive and psychomotor performance, impairments in mental arithmetic and other functions have been found by Schulte (1963) at 5 percent COHb, with some tendency for disruption as low as 2 percent. Beard and his co-workers have shown decreased auditory discrimination of tone length with COHb levels of approxi-

mately 4 to 5 percent (Beard and Wertheim, 1967). On the other hand, Dorcus and Weigand (1929) found no decrements in cognitive functioning or psychomotor tasks with COHb levels as high as 25 to 35 percent. A recent study similarly found no decrement in psychomotor performance, and no disruption of the subjective time estimate of a ten-second interval with COHb level as high as 7 percent (O'Donnell, Mikulka, Heinig, and Theodore, 1970). Stewart et al (1970) found no significant performance effects in males exposed to 100 ppm CO for 9 hours. Obviously, additional work is needed to clarify the reasons behind these apparently contradictory results.

The present study represents an attempt to address this question from both a traditional and novel viewpoint. In addition to a battery of psychomotor tasks, all of which have been used in previous research on CO, a different approach to CO research in performance was introduced on a pilot basis. The subject's sleep patterns were monitored continuously during an all-night exposure to CO, and data were analyzed to determine whether the exposure had any effect on these sleep patterns. Sleep patterns have been shown to be extremely sensitive to central nervous system conditions, and to change markedly with small variations from the normal (Foulkes, Metcalf, and Stoyva, 1968; Foulkes and Hobson, 1969). The existence of normal sleep patterns is dependent on several important structures of the brain, including the reticular formation, limbic system, hypothalamus, thalamus, and, importantly, the cerebral cortex (Zanchetti, 1967; Zung, 1970; Roffwarg, Muzio and Dement, 1966). Biochemically, it appears that the various phases of the sleep-wakefulness cycle depend on the relative concentrations of serotonin and norepinephrine, and/or related substances (Torda, 1969). In view of these facts, it would seem that if CO has any effects on the central nervous system which would be severe enough to show up as performance decrements, then these effects should certainly manifest themselves in alterations of the subjects' sleep patterns. Since this approach had never been tried before, and since the monitoring of sleep patterns is a complex and experimentally time consuming procedure, it was decided that a pilot study would be done using the smallest number of subjects usually necessary to uncover practically important effects on sleep patterns.

## PROCEDURE

### Subjects

Four volunteer male subjects were used in the present study. All subjects were members of the U. S. Air Force, all had undergone altitude training, and all had a currently validated class III flight physical. Subjects' ages were 20, 24, 24 and 42. All of the subjects were known to be non-smokers, and baseline carboxyhemoglobin (COHb) determinations supported this observation.

### Environment

All CO and control exposures were carried out in the Thomas Domes of the Toxic Hazards Division, Wright-Patterson Air Force Base, Ohio. Each dome is a completely

closed environmental system into which a given contaminant can be introduced and maintained within  $\pm 3$  percent of desired concentration. Air flow during the present study was controlled at 20 ft<sup>3</sup>/min. The dome is circular with a diameter of 12 feet and a total volume of 828 ft<sup>3</sup>. CO level was continuously monitored by a non-dispersive infrared analyzer. Temperature was maintained between 68 and 74 F, and in order to maintain a perfect seal on the dome, a slight negative pressure (680 mm Hg) was held throughout all exposures. Subjects entered the dome through an airlock which allowed the interior environment to remain stable.

### Performance Measures

Critical flicker fusion (CFF) was measured by having the subject binocularly and centrally view a red test light 0.375 inch in diameter from a distance of approximately 16 inches. Using the method of limits, the subject adjusted the flicker rate of the light until it just appeared to be a steady light. The actual flicker rate was measured by a Monsanto Model A-100 digital frequency counter. A total of 12 CFF readings were obtained in this way each day for each subject.

The subject's ability at mental arithmetic was measured by using the "solar radiation" test from the School of Aerospace Medicine Neptune Battery. This test, and all subsequent Neptune tests are explained in detail by McKenzie, White and Hartman (1969). Basically, the subject is required to add four one-digit numbers, to add the two digits of that answer. Scoring is in terms of the time necessary to correctly solve a problem, as well as the errors for each problem. In the present study, a total of 20 problems were given to the subject each day.

In order to test the subject's ability to perform tasks under varying workload conditions, two other tasks from the Neptune Battery were used. In one set of tests, referred to as "moderate" workload tests, the subject was required to track a needle dial and to keep it centered within small limits. A sine-wave forcing function was fed into the dial, producing essentially a first-order tracking task. While performing this task, the subject was also required to monitor three other dials located above the tracking dial. At eight randomly selected times during the tracking trial, one of these dials would go off-center. The subject was required to press the correct one of six buttons to return the dial to center. One trial on this moderate workload test lasted one minute, and two trials were obtained each day. The tracking task was scored in terms of total time off target. For the monitoring task, scoring was in terms of the total time taken to see and respond to the offset dials.

Another group of tasks was referred to as the "high workload" test. The same two tasks from the Neptune battery were used, but in addition, the subject was required to simultaneously perform another task. Essentially, he had to monitor three lights which flashed in random order. His task was to note and remember how many times each of the lights flashed during the one-minute tracking trial. At the end of the trial,

he was required to indicate his answer for each light. This test was used solely to add an additional workload on the subject, and was not scored. However, subjects were not informed of this fact, and were instructed to give equal attention to all the tasks in each test.

Because some past studies have indicated that one of the effects of CO exposure is a marked change in the individual's subjective time estimate, three tests were included to measure the subject's ability to estimate time in various ways. In the first of these, the procedure used in a previous study (O'Donnell, Mikulka, Heinig and Theodore, 1970) to determine the subjective estimate of "empty" ten-second time interval was used. In this test, the subject pressed a button each time he estimated that ten seconds had elapsed. These estimates were carried out for two three-minute periods each day. In the second test of this series, the subject was required to hold the button down for an estimated 30 second interval, then release it. He then began another 30 second estimate at will. Four such estimates were obtained twice each day for each subject. The last time estimation task required the subject to compare the length of two tones presented in rapid sequence. The tones were 1,000 cycle pure tones presented to both ears at  $85 \pm 2$  db. The first, or standard tone, was always 1.00 seconds in length, and the second tone varied between 0.675 and 1.325 seconds in 0.025 second intervals. The interval between tones was 0.5 second. For each test, 27 determinations were made by the method of constant stimuli, with 9 comparison tones longer, 9 shorter, and 9 equal to the standard tone. Two such tests were given to each subject each day.

### Sleep Measures

The procedures recommended in the standardized manual for recording and staging sleep (Rechtschaffen and Kales, 1968) were followed in the present study. Beckman bio-potential electrodes were attached to the shaved scalp at the C<sub>3</sub> and C<sub>4</sub> positions for electroencephalographic (EEG) recording. Both of these electrodes were referred to the left mastoid. Grass silver cup electrodes were placed at the outer canthus of each eye and referred to the left mastoid to obtain two channels of electro-occulographic (EOG) records. Two Grass silver cup electrodes were placed on the muscle areas on and beneath the chin (mental-submental) to obtain a single channel of electromyographic (EMG) records. Finally, for medical monitoring purposes, two electrodes were placed on the chest to obtain a single channel of electrocardiogram. Wires from all electrodes were bundled at the top of the subject's head and plugged into an electrode board near the bed. No subject reported unusual discomfort with this electrode placement, and except for two instances where electrodes came loose during the night, recording of the physiological measures was uneventful.

Signals were recorded on a Grass Model 78 recorder. Records were printed out continuously at 15 mm/sec. Scoring was done independently by two trained scorers using the criteria set forth in the standardized manual (Rechtschaffen and Kales, 1968). Agreement between the scorers on a page by page basis, including all stages separately, was between 90 and 95 percent. For the sleep data presented here, the scoring of the author was used.

### Design and Testing Procedures

Each subject slept for 9 nights in the Thomas Dome. The first four nights were used to adapt the subject to the environment and to insure that all baselines were stable. This procedure was necessary since there is a strong "laboratory" effect on sleep patterns which, under normal circumstances, takes several nights to stabilize. In the relatively high noise environment of the dome, in a situation where the subject might have some apprehension, it was necessary to use four complete nights for such adaptation. Beginning on the fifth night, a double-blind procedure was instituted. Neither the subjects, nor experimenters, nor technicians working in the area knew whether CO was present on any given night. Only the chemist-monitor, located remotely from the dome, was aware of the environmental status.

For the five experimental nights, two exposure nights at either 75 or 150 ppm of CO were given. After each exposure night, a "blank" night with no CO present was given. This was done to insure that there would be no residual effect on the dependent variables from the CO exposure. Finally, a control night at 0 PPM of CO was given. Thus, no CO night was followed by another CO night, nor was a CO night followed by the control night.

Subjects entered the dome at 11 o'clock each evening. Electrode placement and final calibration took about 10 minutes, and the lights in the dome were usually turned out by 11:15. Sleep monitoring was begun from that point and continued, uninterrupted, until the subject was awakened at 6 A.M.

After waking the subject, approximately 30 minutes were spent in removing electrode wires, eating, etc., while the contaminant level was maintained. The subject then began the series of performance tests which were all located in the dome.

The first test given was the CFF test. This was followed by mental arithmetic, comparison of tone lengths, the moderate workload Neptune test, 10 second time estimate, the high workload Neptune test, and 30 second time estimate. The subject was then given a 5-minute rest, and the entire series of tests was repeated in reverse order. Total testing time was approximately 1 1/2 hours, so that the series ended at approximately 8 A.M. At this point, 10 ml of blood was taken for COHb, hematocrit and hemoglobin determination. The subject was then removed from the dome, taken to an adjoining room, and required to breathe 100 percent oxygen for one hour.

## RESULTS

Carbon Monoxide Exposure Levels

Carboxyhemoglobin (COHb) determinations were made on the venous blood for each subject after each experimental session. These determinations were done by a modified gas chromatographic method of Dominguez, Christensen, Goldbaum and Stembridge (1959). The results of the COHb analyses are presented in table I. From this table, it can be seen that the COHb levels reached after approximately nine hours of exposure reflect a direct relationship with the level of ambient CO. It should be pointed out, however, that these values are somewhat lower than those which would be predicted from the use of theoretical curves generally available. However, these curves usually assume a relatively high ventilation and pulse rate, whereas during sleep these values are usually quite low. This would account for the unexpectedly low COHb levels found here.

TABLE I  
FINAL CARBOXYHEMOGLOBIN LEVEL (PERCENT) AT EACH EXPOSURE LEVEL

SUBJECT	0 PPM	75 PPM	150 PPM
1	0.5	6.8	11.9
2	0.5	5.1	12.9
3	0.7	6.4	14.3
4	0.8	5.2	11.6
MEAN	0.6	5.9	12.7

Performance Measures

Time Estimation: In order to test the hypothesis that low level exposure to CO affects the estimate of subjective time, three different tests were administered. In the first test, the subject was required to estimate the passage of 10 second intervals by pressing a button, releasing it, and waiting the estimated amount of time before repeating the procedure. In the second test, the subject was required to hold the button down to estimate four 30-second intervals. In the third test, the subject was required to determine whether the second of two 1,000 cycle tones was longer or shorter than the first or standard tone.

Data on these three tests are presented in table II. With respect to the 10-second estimates, it can be seen that the interval was overestimated under all conditions. None of the differences was significant, and the only possible trend appeared to indicate that estimates were becoming more "accurate" under the CO conditions. Inspection of the data from individual subjects revealed that in 3 out of the 4 cases under each CO condition, the estimates were closer to the 10-second "real" time than under the control condition. For the 30-second estimates, it is again seen that no significant differences appeared between the control and CO groups. Further, no consistent trend appeared other than overestimation under all conditions. Individual subject's estimates appeared to follow no pattern related to the presence or absence of CO.

TABLE II  
TIME ESTIMATION AFTER EXPOSURE TO CO CONDITIONS

TEST		0 PPM	75 PPM	150 PPM
10 Second Estimate (Seconds)	MEAN	11.43	10.58	10.38
	SD	1.72	1.51	1.25
30 Second Estimate (Seconds)	MEAN	35.59	35.82	34.51
	SD	5.32	5.25	5.97
Comparison of Tone Lengths (Errors)	MEAN	6.37	6.13	6.50
	SD	4.22	2.30	1.54

The tone comparisons were scored simply as correct or incorrect responses. As can be seen from table II, the mean differences between groups in the number of errors were small and did not reach statistical significance in any case. There were slightly fewer errors under 75 ppm CO than under the control condition, and slightly more errors under 150 ppm than under the control condition. Individual subjects' estimates revealed an almost random pattern with respect to CO condition, with 5 of the 8 CO exposures producing fewer errors than their respective controls. In summary, then, it can be seen that the present study cannot support the hypothesis that CO at these exposures produced a decrement in time estimation. Further, from the present data, there is no reason to suspect a trend to such an effect.

Critical Flicker Fusion (CFF): In addition to the above tests, a test of CFF was given to the subject. Each subject made 12 determinations of the CFF point each day and the mean of these determinations was calculated as his CFF point. These individual means were then combined to form the group means which are presented in table III. Again, it can be seen that none of the differences were significant, and that no consistent trend toward lower CFF values was seen as a function of CO exposure. Looking at individual subjects' data, no trends were apparent. In two of the four cases under each CO condition, subjects had higher (better) CFF scores than under the control condition. It therefore must be concluded that the present study failed to find any effect of CO on critical flicker fusion.

TABLE III  
CRITICAL FLICKER FUSION AFTER EXPOSURE TO CO CONDITIONS

TEST		0 PPM	75 PPM	150 PPM
CFF				
(Cycles Per Second)	MEAN	40.60	40.30	40.80
	SD	5.78	4.73	6.16

Cognitive and Psychomotor Tests: The final series of performance tests administered to each subject was designed to probe a complex of cognitive and psychomotor functions. In the first of these tests, the subject was required to perform 20 arithmetic calculations, each involving additions and multiplications, and calling for use of short term memory. In addition to this test, two tests involving identical tasks but under different workloads were presented to the subject. In one case, he was required to track a needle dial to keep it centered, while a sine wave forcing function offset the dial. At the same time, he was required to monitor three dials and to correct any dial which went off center by pressing one of six switches. The scores for these two tests were the total time off target for the tracking task, and the cumulative time to respond to the dials in the monitoring or vigilance task. In the second test of this series, the subject was required to perform the same two tasks as above. At the same time, he was required to watch three lights and keep track of the number of times each light flashed during the trial. This task was not scored, since it was included simply to add an additional workload on the subject. Subjects had been instructed to give equal attention to all the tasks.

The results of these three tests are presented in table IV. Looking first at the mental arithmetic task, it can be seen that the total mean time to solution was slightly less under 75 ppm and slightly more under 150 ppm than under the control condition. In neither case were the differences significant. For the individual subjects, 7 of the 8 CO exposures produced better mental arithmetic scores than their respective controls. It therefore seems clear that for this extremely complex mental arithmetic task, exposure to CO for these durations had no detrimental effect.

TABLE IV  
COGNITIVE FUNCTION AFTER EXPOSURE TO CO CONDITIONS

TEST		0 PPM	75 PPM	150 PPM
MENTAL ARITHMETIC (Time to Solution in Seconds)	MEAN SD	92.38 15.73	89.78 15.82	98.60 17.33
<b>MODERATE WORKLOAD</b>				
Tracking (Time off target, sec.)	MEAN SD	20.40 6.29	17.26 6.41	23.17 8.15
Vigilance (Cum. time to action, sec.)	MEAN SD	22.90 2.55	27.60 9.48	23.15 3.34
<b>HIGH WORKLOAD</b>				
Tracking (Time off target, sec.)	MEAN SD	27.73 5.64	22.40 5.09	25.00 5.19
Vigilance (Cum. time to action, sec.)	MEAN SD	37.30 9.63	42.90 14.33	31.40 11.55

Considering now the tasks listed under "moderate workload" it can be seen that again none of the comparisons for either task was statistically significant. For the tracking task, performance was slightly better under 75 ppm CO, and slightly worse under 150 ppm CO than under the control condition. The vigilance scores similarly showed no significant differences as a result of CO exposure. Under both CO conditions the mean scores were slightly worse than under the control condition; however, inspection of the scores for individual subjects revealed that in 5 of the 8 cases, performance was better under CO than in the control condition.

In the tasks listed under "high workload", tracking was slightly better under both CO conditions. Vigilance was worse under 75 ppm exposure and better under 150 ppm. Again, none of these differences was statistically significant.

It is interesting to note the effect of the additional task in the "high workload" tests. It can be seen by comparing identical tasks under both conditions that, as would be expected, the high workload condition produced uniformly worse scores. For the vigilance task, this effect was particularly noticeable, resulting in a mean total of 12.65 additional seconds to respond during the high workload condition. The difference in vigilance score between the two workload conditions was significant at the .05 level. This indicates that the addition of one task to a battery of two tasks as used here resulted in a greater effect on performance than all of the CO used in the present study.

### Sleep Measures

The primary analyses planned for the sleep data in the present study were designed to answer the question of whether there was a change in the overall sleep pattern of subjects under carbon monoxide. These data will be presented here, while more intensive analyses of specific questions relating to the subjects' sleep will be presented at a later date. Figure 1 presents the mean percentage of total sleep time spent in each of the stages of sleep, along with the  $\pm 1$  SD bar. Obviously, none of the differences between the control group and either CO group, or between the two CO groups, are significant. Inspection of the figure reveals that the differences between groups which do appear are so small, even in absolute terms, as to be negligible.

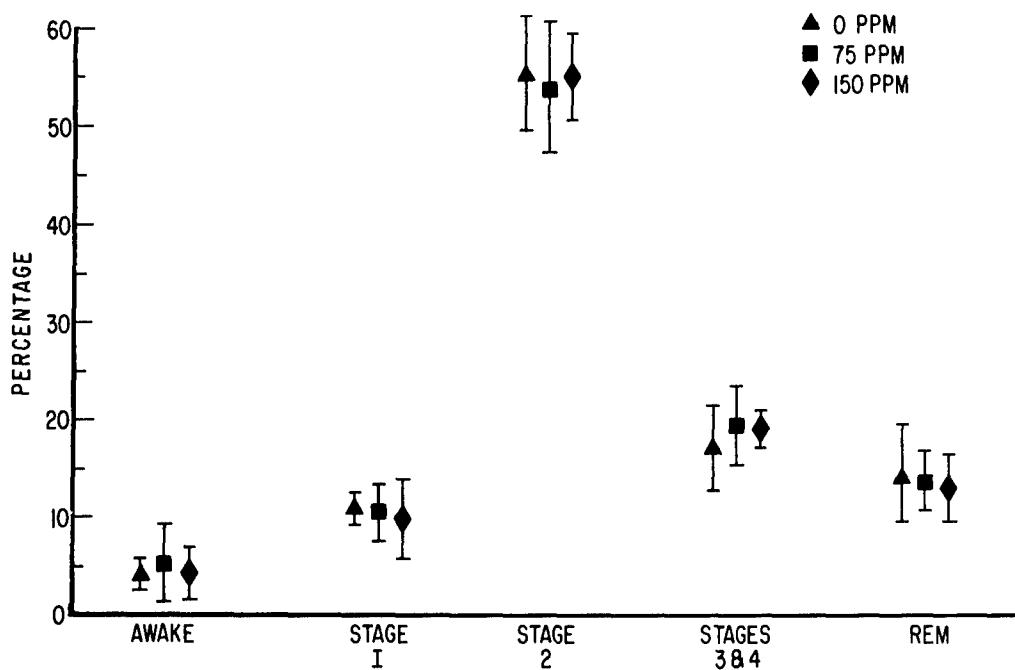


Figure 1. PERCENTAGE ( $\pm 1$ SD) OF TOTAL SLEEP IN EACH STAGE (WHOLE NIGHT).

In order to further determine whether there was a change in the overall sleep pattern due to CO exposure, the last three hours of the night were looked at separately. By this time, the subjects' COHb levels had reached a high point, and presumably any effects which would show up would be most evident. The data for these time periods are presented in figure 2. Although none of the comparisons was significant, there does appear to be some separation between the CO and control groups in stages 1 and 2. The data for individual subjects revealed no consistent pattern for stage 2 sleep, but subjects in all eight CO exposures showed less stage 1 sleep than they had in the control condition.

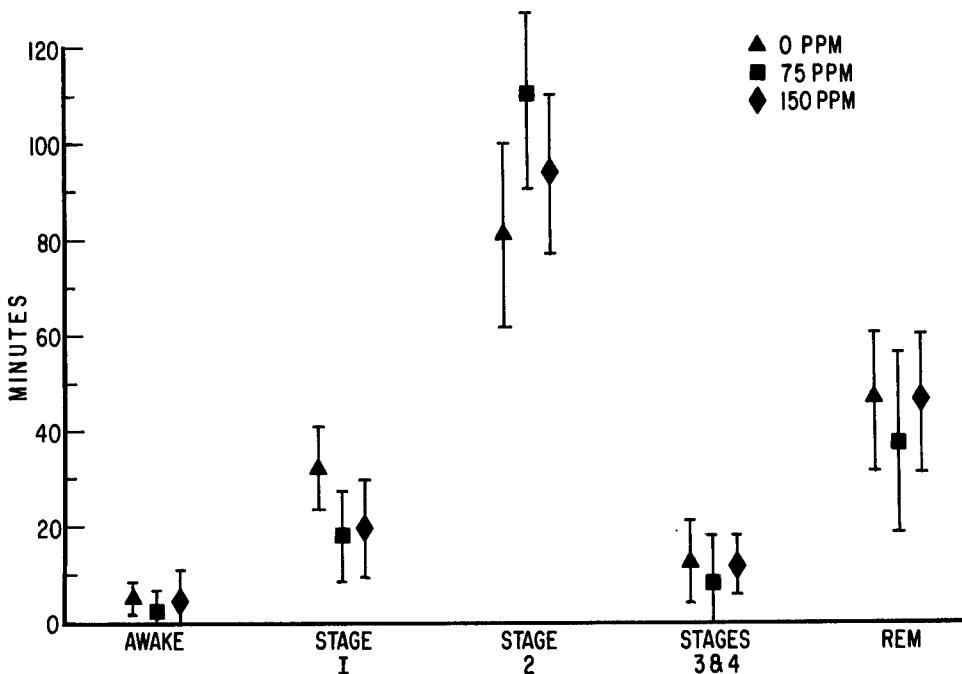


Figure 2. MINUTES ( $\pm 1SD$ ) SPENT IN EACH STAGE OF SLEEP DURING LAST THREE HOURS OF CO EXPOSURE.

It is possible that CO exposure could have affected sleep in ways which would not be evident from an analysis of the total pattern. For this reason, several other analyses were done on the sleep data. In the first of these analyses, the mean number of times subjects entered the various stages during the course of the night were compared under differing CO conditions. These data are presented in table V. Again, none of the comparisons was significantly different between exposure conditions. It was noted, however, that in every stage of sleep, there were fewer stage changes in both CO conditions than there were in the control condition. This would provide an extremely tentative indication that there may have been slightly less "mobility" or restlessness under CO than in the control condition.

TABLE V  
MEAN NUMBER OF TIMES IN EACH SLEEP STATE  
DURING CO EXPOSURE CONDITIONS

STAGE		0 PPM	75 PPM	150 PPM
Awake	MEAN	6.00	4.75	4.50
	SD	2.35	2.28	3.20
1	MEAN	19.00	17.50	14.75
	SD	4.64	7.63	6.14
2	MEAN	18.25	17.00	17.00
	SD	2.49	4.06	2.55
3&4	MEAN	10.50	10.25	9.00
	SD	1.66	4.09	1.87
REM	MEAN	9.75	8.50	9.00
	SD	5.40	4.33	4.74

In order to probe this possible effect further, the mean duration of each stage of sleep during the night was determined. Thus, the total amount of time spent in each stage was divided by the number of times the subject entered that stage. These data are presented in table VI. None of the comparisons was significantly different from each other. There was a trend toward longer times spent awake and in stages 1, 2, and 3, under the CO conditions, and slightly shorter times spent in stages 4 and REM under CO. This result would indicate that if the previous indication of less mobility under CO is correct, than this is achieved by spending longer periods of time in the lighter stages of sleep. Obviously, lacking statistical significance, no definitive statements can be made. However, the above observations warrant further study.

TABLE VI  
MEAN DURATION OF SLEEP STATES (IN MINUTES)  
DURING CO EXPOSURE CONDITIONS

STAGE		0 PPM	75 PPM	150 PPM
Awake	MEAN	2.70	3.75	4.85
	SD	.66	1.80	4.25
1	MEAN	2.30	2.43	2.58
	SD	.44	.48	.19
2	MEAN	11.60	12.85	12.68
	SD	1.16	3.64	2.03
3	MEAN	5.30	7.00	7.10
	SD	1.50	4.10	3.04
4	MEAN	12.00	10.90	11.00
	SD	10.00	4.42	3.06
REM	MEAN	9.35	8.75	7.68
	SD	5.86	3.35	2.60

### CONCLUSIONS

It would appear that when subjects are allowed to sleep for a normal period of time in the presence of CO at a level up to 150 ppm, there is no major disruption of either their sleep patterns or subsequent psychomotor performance involving time estimation, mental arithmetic, tracking, or vigilance under either moderate or high workloads. With respect to the performance measures, no patterns were isolated which would indicate that more detailed study under the same conditions would yield any significant effects of CO exposure. Some extremely tenuous indications of possible changes in the mobility of subjects during their early stages of sleep were uncovered, and these should be investigated further.

### ACKNOWLEDGEMENTS

Special appreciation is due to Drs. B. O. Hartman, G. V. Pegram, and M. Ohlbaum for extremely helpful consultations on various aspects of this study. In addition, the study could not have been carried out without the technical support of the SysteMed Corporation, or of Mr. B. C. Dixon of the Lear-Siegler Corporation. An efficient chemical toilet for use in the dome was supplied by Koehler-Dayton, Inc., 401 Leo Street, Dayton, Ohio, 45404.

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## DISCUSSION

MR WANDS (National Academy of Sciences): What was the average duration of time after entry until the carboxyhemoglobin level was determined? They entered at 11 o'clock, I believe you said, but I did not catch the exit time.

CAPTAIN O'DONNELL (Aerospace Medical Research Laboratory): It was approximately 8 o'clock in the morning, so it would be approximately nine hours exposure. The level was taken at the end of exposure.

DR. BEARD (Stanford University Medical Center): What was the noise level in the domes?

CAPTAIN O'DONNELL: The noise level was fairly high, I think it's around 84 db, 85 db. Out of the cone it would be less.

DR. MAC EWEN (SysteMed Corporation): Our dome tops act as parabolic reflectors of noise. The noise in the center of the dome is 85 db and in some sections is perhaps even higher. At the periphery, where the man in the bed was, the noise level was probably 60 to 65 db based on previous measurements made under these conditions.

CAPTAIN O'DONNELL: I might mention that at first we asked the subjects very thoroughly whether the noise seemed to bother their sleep and, subjectively, none of the subjects reported that the noise bothered their sleep beyond the first night. This was one of the reasons why we gave four nights of adaptation to the dome. Sleep obviously is very sensitive to changes in location and we wanted to give enough nights so that the subject would be completely stabilized and, in fact, that happened within about three nights.

DR. AZAR (E. I. duPont de Nemours and Company): I was wondering if you looked into any effect of learning on your psychomotor tests because it appeared that on several instances that under carbon monoxide the scores tended to improve, and if your subjects were learning the psychomotor tests and then you compared test scores with their control levels, if there was a subtle effect of carbon monoxide you wouldn't have picked this up unless you looked at a learning trend.

CAPTAIN O'DONNELL: There would be two things on that. The first thing is that we tested the subjects on the tests every day which means they had nine nights of practice, and that they stabilized by the fourth night. They were all relatively simple, easily learned tests--they were included for that reason. By the fourth night, which would be four hours of practice, they were stabilized. And the second thing is that this was counterbalance design. So any learning effects should be balanced out. In other words, the carbon monoxide was not always the last exposure.

DR. AZAR: I meant in looking and analyzing your data, did you negate the learning phase?

CAPTAIN O'DONNELL: Only to the point of assuring that there was a plateau prior to the exposure starting. In other words, we did look to the extent that we assured ourselves that the subject had plateaued in performance and that he was not continuing to learn before we started exposures.

DR. HODGE: If I understand, after the four nights of practice, which was uniform for all subjects, and thereafter some subjects had carbon monoxide the first night, some the second or third?

CAPTAIN O'DONNELL: Yes, there was completely random choice out of whatever method they chose to randomize so that, I forgot the exact sequencing, but I think one subject had carbon monoxide on the first night, two subjects had a control on the first night, and it was completely random among the four subjects.

FROM THE FLOOR: What were smoking habits in the subjects?

CAPTAIN O'DONNELL: We assured ourselves because of some previous experience that none of the subjects were smokers, both by observation and by carboxyhemoglobin determinations under control conditions. We had one subject in the previous study that lied to us. We caught him on the carboxyhemoglobin.

DR. ROBERTSON (Hazleton Laboratories, Inc.): I wondered whether in addition to your scientific observations you made any notes on mood or such factors as impetuosity or clumsiness? Whether there is any change indicating loss of inhibition in subjects?

CAPTAIN O'DONNELL: We had the subjects fill out a questionnaire after each exposure and we also questioned them the night following each possible exposure as to their behavior during the day and we got absolutely no subjective response. There was no subjective ability to determine whether carbon monoxide was present, there were no consistent reports of any changes. Twice we thought we had beaten the double blind, since subjects reported that they went home and slept for three or four hours--two different subjects said this "I went home and slept today". And I thought, well this must be it, and they both turned out to be zero conditions. There was absolutely no correlation between subjective report and what we later found out to be CO exposure.

DR. HODGE: I remember you said in your checking of these average numbers that you found no statistical significance, even where there were consistent differences, always small, but always the same direction. Now did you do a sign test check on this, and was that not significant also?

CAPTAIN O'DONNELL: That's correct with one exception of the stage one sleep where we had eight positive changes. All of the apparent mean differences here were also checked nonparametrically and none of them was significant with that one exception.

EXPERIMENTAL HUMAN EXPOSURE TO CARBON MONOXIDE  
< 1 TO 1000 PPM\*

Richard D. Stewart, M.D., M.P.H.  
•  
Jack E. Peterson, Ph.D.  
Michael J. Hosko, Ph.D.  
Edward D. Baretta  
Hugh C. Dodd  
Paul E. Newton  
Terrance N. Fisher, M.D.  
and  
Anthony A. Herrmann, M.D.

The Medical College of Wisconsin  
Milwaukee, Wisconsin

As man grapples with the problems of air pollution in an attempt to establish air quality standards, he finds he possesses limited information regarding the biological effects of short-term and continuous exposure to low concentrations of carbon monoxide (CO). Only a few investigations have been conducted in which sophisticated instrumentation was used to search for minute changes in human performance induced by exposure to low concentrations of CO (McFarland et al, 1944; Schulte, 1963; Beard and Wertheim, 1967). Lacking is the information as to whether these minor alterations in function which have been reported are of any practical significance to the health, performance, or judgment of man as he performs his daily tasks.

EXPERIMENTAL PROCEDURE

In an attempt to gather additional human toxicological information about CO, a series of experimental exposures to known concentrations of the gas was conducted. These exposures were designed to simulate the type encountered for industry and in urban areas where CO is absorbed over a period of hours.

\*Most of the information in this report has been published in the Archives of Environmental Health, 21:154-180, August, 1970.

### Exposure Chamber

A room measuring 20 x 20 x 9 feet high served as the exposure chamber. The air flow through the room to the exhaust was 500 cu ft/min, which created a slight negative pressure within this chamber. Carbon monoxide was continuously metered into the chamber from a compressed gas cylinder in the adjacent command laboratory. The CO used was a chemically pure grade with a minimum purity of 99.5%.

The air-conditioned chamber featured pleasant lighting, comfortable chairs, and study desks. Activity within the chamber was strictly sedentary. Meals were served to the subjects during the exposures, and coffee and soft drinks were available on an ad lib basis. The subjects were under continuous visual surveillance by medical personnel while in the chamber. In addition, all chamber activities were visually monitored and video taped in the command laboratory by closed circuit TV.

### Analysis of Exposure Chamber Atmosphere

The concentration of CO in the chamber atmosphere was recorded continuously by an infrared spectrometer equipped with a 10-meter pathlength gas cell which was continuously flushed with air drawn from the chamber through 1/4-inch diameter polyethylene tubing.

The chamber atmosphere was also monitored periodically by a gas chromatograph (GC) equipped with a helium ionization detector. Calibration standards of CO and air were prepared in saran bags and were analyzed by both the infrared and gas chromatographic methods before and every hour during each experiment.

### Subjects

Twenty-eight healthy medical students and medical school faculty ranging in age from 24 to 42 years volunteered for the exposure studies. Only three were smokers, but they agreed to abstain from smoking for the duration of the study. Each was given a comprehensive medical examination which included a complete history and physical examination and the laboratory studies listed below.

### Exposure Schedule

Table I-A presents the exposure schedule and lists the concentration and duration for the first 25 experiments. Experiments 1 and 9 were control experiments. The CO concentrations in experiments 2 through 8 and 10 through 13 were selected at random so that neither the volunteers nor the investigators who conducted the testing procedures knew the concentration of CO on a given test day. The same group of subjects was in experiments 1 through 8 while a different group was studied in experiments 9 through 13. Two toxicologists from the medical school faculty were the subjects in experiments 22 through 25.

TABLE I-A  
HUMAN EXPOSURE TO CARBON MONOXIDE

<u>Experiment</u>	<u>No. of Subjects</u>	CO Concentration, ppm			<u>Duration (hr)</u>
		<u>Mean</u>	<u>SD</u>	<u>SE</u>	
1	8	< 1	...	...	8.0
2	7	< 1	...	...	8.0
3	8	26.4	1.2	0.20	8.0
4	7	103.2	6.9	0.90	8.0
5	8	24.8	1.3	0.20	8.0
6	7	1.6	0.3	0.04	8.0
7	8	49.4	7.5	0.90	8.0
8	7	98.0	2.8	0.40	8.0
9	4	< 1	...	...	8.0
10	4	< 1	...	...	8.0
11	4	94.0	5.7	0.7	8.0
12	4	< 1	...	...	8.0
13	4	99.8	6.0	0.8	8.0
14	9	51.6	0.5	0.2	1.0
15	6	51.2	1.0	0.2	3.0
16	3	50.3	1.5	0.2	8.0
17	3	49.0	1.8	0.2	24.0
18	10	99.7	2.1	0.8	1.0
19	6	98.1	2.4	0.5	3.0
20	2	100.2	3.2	0.4	8.0
21	11	199.5	9.9	1.6	4.0
22	2	507.0	17.3	3.5	1.8
23	2	494.0	25.4	3.4	2.3
24	2	473.3	27.4	3.6	2.3
25	2	598.0	Range: 1 ppm rising to 1000 ppm		

TABLE I-B  
TIME DISCRIMINATION - TIME ESTIMATION EXPERIMENTS

<u>Experiment</u>	<u>No. of Subjects</u>	CO Concentration, ppm			<u>Duration (hr)</u>
		<u>Mean</u>	<u>SD</u>	<u>SE</u>	
26	8	100.99	4.28	0.54	5.0
27	8	50.64	3.83	0.54	5.0
28	8	196.49	3.00	0.41	5.0
29	8	< 2	...	...	5.0
30	8	49.82	2.36	0.31	5.0
31	7	201.38	6.58	0.95	5.0
32	4	4.44	0.76	0.06	24.0
33	6	< 2	...	...	5.0
34	7	99.81	3.77	0.77	2.5
35	8	96.13	4.39	0.62	5.0
36	6	203.69	6.39	1.33	2.5
37	4	< 2	...	...	24.0
38	8	< 2	...	...	5.0
39	4	24.36	2.24	0.17	24.0
40	6	< 2	...	...	2.5
41	6	49.45	1.43	0.27	2.5
42	6	201.70	4.21	0.72	2.5
43	6	< 2	...	...	2.5
44	6	49.67	3.56	0.66	2.5
45	4	99.93	1.66	0.31	2.5
46	2	201.72	6.94	1.36	2.5
47	2	192.90	5.85	1.15	2.5
48	2	< 2	...	...	2.5
49	2	< 2	...	...	2.5
50	2	< 2	...	...	2.5
51	6	< 2	...	...	5.0
52	2	193.1	13.4	2.6	2.5
53	2	197.7	9.56	1.69	2.5
54	2	< 2	...	...	2.5
55	6	192.3	21.4	3.5	5.0
56	2	199.96	2.97	0.56	2.5
57	2	< 2	...	...	2.5
58	2	< 2	...	...	2.5
59	5	196.98	8.66	1.69	5.0

Table I-B presents the exposure schedule for the series of experiments designed to investigate the effect of CO upon time estimation and time discrimination.

### Clinical Testing

A preexposure venous blood sample was obtained for a complete blood cell count, sedimentation rate, sodium, carbon dioxide, chloride, potassium, calcium, total serum protein, alkaline phosphatase, bilirubin, blood urea nitrogen, glucose, serum glutamic oxaloacetic transaminase, and carboxyhemoglobin determination. This battery of blood tests was repeated 16 hours after each exposure to CO which featured concentrations of 100 ppm or greater. Baseline values for the following tests were obtained: hand and foot reaction time in the American Automobile Association (AAA) driving simulator, Crawford collar and pin test, Crawford screw test, hand steadiness in the AAA steadiness test, Flanagan coordination test, othorator visual test, complete audiogram, resting 12-lead ECG, standard electroencephalogram, visual evoked response (VER), 10-second time estimation, 30-second time estimation, Beard-Wertheim time discrimination test (Beard, 1967), and time estimation-hand reaction time test. Those subjects exposed to concentrations of CO in excess of 100 ppm had first to demonstrate a normal exercise ECG which monitored the effect of vigorous running in place for a three-minute interval. The orthorator visual test included an evaluation of far vision vertical and lateral phoria, far vision acuity, depth perception, color vision, near vision acuity, and near vision vertical and lateral phoria.

Each subject was given a repeat physical examination one hour before entering the exposure chamber. At this time he was queried as to whether he was experiencing headache, nausea, dizziness, abdominal pain, chest pain, eye, nose or throat irritation, or any other subjective symptom. Venous blood and alveolar breath samples for analysis completed the preexposure evaluation.

During the exposures, the subjective and objective responses of each individual were recorded during every waking hour. In experiments 1 through 13, physiological performance tests were periodically conducted and always within the final hour of CO exposure. In the remaining experiments, selected tests were performed. Venous blood samples for carboxyhemoglobin (COHb) and total hemoglobin analysis were obtained serially from each subject after he has passed his arm through a small sampling port in the exposure chamber into an uncontaminated atmosphere (figure 1, table II).

Following each exposure, serial venous blood samples were obtained in an adjacent contamination-free laboratory for COHb analysis. At the same time, alveolar breath samples were collected in saran bags, using the 20-second breath-holding technique for infrared analysis and in glass breath collection pipettes (Health Science Services, Brookfield, Wisconsin) for GC analysis (Stewart et al, 1965). All untoward subjective responses occurring in the first 24-hour postexposure interval were recorded.

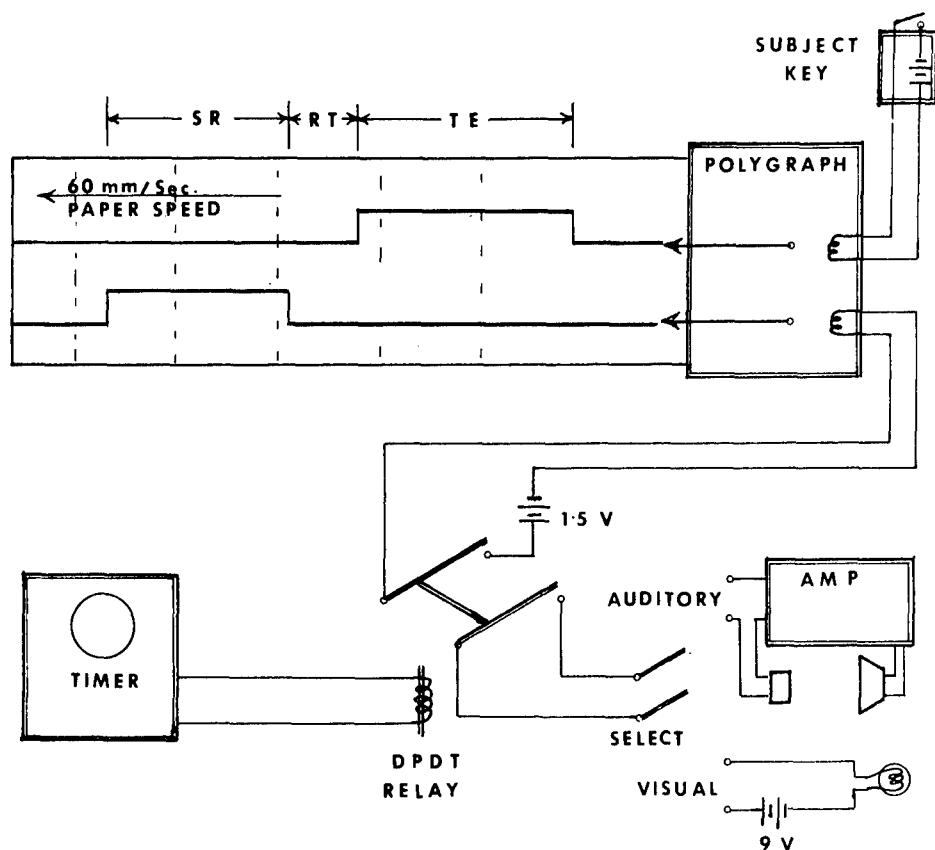


Figure 1. SCHEMATIC DIAGRAM OF REACTION TIME - TIME ESTIMATION APPARATUS. Stimulus Record (SR), Response or Reaction Time (RT), and Subjects Estimate of Stimulus Duration (TE). (Reprinted with permission from the Archives of Environmental Health, 21:156, August, 1970).

TABLE II

PERCENT OF COHB DURING AND FOLLOWING EXPOSURE  
TO 50 PPM OF CO

Time During Exposure	Mean	Range	No. of Subjects
Preeexposure	0.7	0.4-1.5	11
30 min	1.3	1.3	3
1 hr	2.1	1.9-2.7	11
3 hr	3.8	3.6-4.2	10
6 hr	5.1	4.9-5.5	5
8 hr	5.9	5.4-6.2	5
12 hr	7.0	6.5-7.9	3
15 $\frac{1}{2}$ hr	7.6	7.2-8.2	3
22 hr	8.5	8.1-8.7	3
24 hr	7.9	7.6-8.2	3
<hr/>			
Time after 1 hour of exposure			
30 min	1.8	1.8	3
1 hr	1.7	1.6-1.8	3
2 hr	1.5	1.4-1.5	3
5 hr	1.1	1.0-1.1	2
<hr/>			
Time after 3 hours of exposure			
30 min	3.7	3.4-3.9	3
1 hr	3.3	2.7-3.8	3
2 hr	2.7	2.3-3.0	3
<hr/>			
Time after 8 hours of exposure			
30 min	5.6	5.1-5.9	3
1 hr	5.1	4.8-5.4	3
1 3/4 hr	4.0	...	3
11 hr	1.5	1.4-1.7	3
<hr/>			
Time after 24 hours of exposure			
30 min	7.5	7.2-7.8	3
1 hr	6.7	6.4-7.1	3
2 hr	5.8	5.6-6.2	3

### Performance Testing

Time Estimation: In experiments 1 through 13, the time estimation test was performed immediately upon entrance of the subjects into the chamber after four and seven hours of exposure. In exposures of shorter duration, the final test was conducted during the final 30 minutes of exposure. In experiments 26 through 59 the test was performed each hour. This test required approximately seven minutes to perform and consisted of a series of nine tone stimuli followed by nine light stimuli of approximately 1, 3 or 5 seconds' duration presented in a random sequence with three stimuli at each time interval. At the termination of the stimulus, the subject depressed a push button for that interval of time he estimated to be equal in length to the original auditory or light stimulus. This was a measure of his ability to estimate the duration of the stimulus.

Figure 1 is a schematic diagram of this testing apparatus. A PBM 0- to 15-second, synchronous, motor-driven interval timer was used to initiate this stimulus-response sequence. The interval timer energized the coil of a double-pole double-throw relay for a preselected stimulus period. The stimulus period randomly approximated intervals of 1, 3, and 5 seconds. One pole of the double-pole double throw relay was utilized to close a 1.5-v circuit to one pen of a polygraph (Grass Model 6 EEG). This circuit provided a record of the exact duration of the stimulus. The second pole of the relay was connected across a selector switch to provide either a visual or an auditory stimulus. The visual stimulus was the light from a 9-v flashlight bulb diffused through a polyethylene tube to provide comfortable viewing. The auditory stimulus was generated by inducing oscillation in a small transistorized power amplifier (Realistic No. 277-1240-Radio Shack). Oscillation was induced by placing a small crystal microphone close to the output speaker. The tone was keyed on and off by interrupting the speaker circuit. This method provided sharp, crisp, tone initiation and termination.

Each subject was provided with a small aluminum minibox which contained a 1.5-v cell in series with a push-button switch connected to a pen of the polygraph. The record of the stimulus response was recorded by means of the polygraph pen deflection, which could easily be read to the closest 10 msec.

In each case, the subject's estimate of the stimulus duration was divided by the actual stimulus duration to normalize the response. Then three replicate normalized responses for each stimulus type and duration were averaged to obtain a mean normalized response for that exposure. A grand normalized time estimate response was determined for each subject by averaging normalized responses over both types of stimulus and for all exposures to control concentrations.

Responses obtained during exposures to CO were treated in two ways. First, mean normalized responses for each subject were used to obtain an overall average and standard deviation for each type of stimulus and stimulus duration at each exposure duration. Each subject's mean normalized response was subtracted from his characteristic response, and differences were used to determine the paired t for that set of

stimulus and exposure parameters. Data are shown in table IV. Second, mean normalized response data were also used in regression analysis to determine whether exposure duration, CO concentration, stimulus type, or stimulus duration were correlated significantly with response. These data follow the previous data in table V.

Expt.	CO Conc. ppm	n*	Driving Simulator					Hand Steadiness				
			Mean	SD**	Paired t	r	Mean	SD**	Paired t	r	t <sub>0.05</sub>	r <sub>0.05</sub>
Control	2	34	35.3	7.58	...		48.6	9.18	...		...	
3	26.4	8	31.5	5.76	1.36		44.6	9.87	-2.32		2.31	
5	24.8	8	32.1	7.99	1.20		53.9	7.07	-2.64		2.31	
7	49.4	8	32.4	6.12	1.20		53.2	7.14	-2.64		2.31	
4	103.2	7	30.2	4.87	1.12		51.9	6.31	-1.33		2.36	
8	98.0	7	32.4	4.86	0.98		48.0	10.93	-1.12		2.36	
11	94.0	4	31.8	4.27	2.36		46.2	3.30	-2.40		2.78	
13	99.8	4	34.0	6.16	1.24	-0.197	48.0	3.89	-0.28	-0.028	2.78	0.220

\*Number of subjects participating in specific experiments.

\*\*Standard deviation.

Expt.	CO Conc. ppm	n*	Crawford Collar and Pin					Crawford Screw				
			Mean	SD**	Paired t	r	Mean	SD**	Paired t	r	t <sub>0.01</sub>	r <sub>0.05</sub>
Control	2	34	26.9	3.77	...		18.2	4.78	...		...	
3	26.4	8	27.6	5.29	-0.88		19.8	5.11	-1.68		3.36	
5	24.8	8	28.7	2.62	-1.60		19.4	5.76	-1.20		3.36	
7	49.4	8	32.9	3.98	-2.24		19.2	4.50	-1.20		3.36	
4	103.2	7	27.0	4.51	0.00		19.0	5.52	-0.07		3.50	
8	98.0	7	31.5	4.66	-0.49		19.8	5.17	-0.07		3.50	
11	94.0	4	28.3	2.66	-0.88		20.1	4.50	-1.88		4.60	
13	99.8	4	21.0	3.32	-3.84	-0.251	19.6	2.50	-1.68	-0.106	4.60	0.220

TABLE IV  
INFLUENCE OF CO EXPOSURE ON TIME ESTIMATION TEST

Experiment	CO Concentration ppm	Exposure During Hours		Stimulus Type	Duration, sec	n*	Normalized Time Estimate			
							Mean	SD **	Paired t	t <sub>0.05</sub>
Control	< 2		All	All	All	414	0.67	0.101	...	...
7	39.6	0.1	Tone		1	8	0.71	0.101	-.64	2.36
			Tone		3	...	0.59	0.044	.64	
			Tone		5	...	0.63	0.045	-.24	
			Light		1	...	0.62	0.061	.32	
			Light		3	...	0.63	0.041	.16	
			Light		5	...	0.59	0.071	.64	
7	49.0	7	Tone		1	8	0.65	0.149	0	2.36
			Tone		3		0.75	0.079	-.08	
			Tone		5		0.62	0.133	.24	
			Light		1		0.66	0.181	-.08	
			Light		3		0.60	0.158	.40	
			Light		5		0.59	0.135	.56	
11	90.3	0.5	Tone		1	4	0.57	0.127	0.96	3.18
			Tone		3		0.59	0.065	0.84	
			Tone		5		0.65	0.050	0.12	
			Light		1		0.62	0.101	0.48	
			Light		3		0.61	0.136	0.52	
			Light		5		0.57	0.128	1.44	
13	98.0	0.5	Tone		1	4	0.59	0.115	.72	3.18
			Tone		3		0.57	0.050	.32	
			Tone		5		0.61	0.045	0.64	
			Light		1		0.60	0.105	0.64	
			Light		3		0.70	0.048	-0.40	
			Light		5		0.62	0.085	0.44	

TABLE IV (Cont'd)

Experiment	CO Concentration ppm	Exposure During Hours	Type	Stimulus Duration, sec	n*	Normalized Time Estimate			
						Mean	SD**	Paired t	$t_{0.05}$
8	98.2	4	Tone	1	7	0.66	0.137	.49	2.45
			Tone	3		0.67	0.066	.42	
			Tone	5		0.65	0.041	.56	
			Light	1		0.69	0.109	.35	
			Light	3		0.64	0.063	.63	
			Light	5		0.57	0.162	1.33	
11	94.4	8.6	Tone	1	4	0.56	0.075	1.48	3.18
			Tone	3		0.58	0.150	0.92	
			Tone	5		0.64	0.022	0.28	
			Light	1		0.64	0.147	0.12	
			Light	3		0.60	0.047	1.00	
			Light	5		0.62	0.000	0.48	
8	98.2	7	Tone	1	7	0.72	0.101	.07	2.45
			Tone	3		0.57	0.126	1.05	
			Tone	5		0.64	0.066	.33	
			Light	1		0.61	0.151	0.77	
			Light	3		0.69	0.064	0.20	
			Light	5		0.62	0.125	0.77	
13	99.8	9	Tone	1	4	0.61	0.155	0.36	3.18
			Tone	3		0.60	0.047	0.72	
			Tone	5		0.63	0.040	0.40	
			Light	1		0.70	0.141	-0.36	
			Light	3		0.71	0.040	-0.48	
			Light	5		0.61	0.138	0.48	

\* Number of subjects participating in specific experiments.

\*\* Standard deviation.

Ten- and Thirty-Second Time Estimation: In experiments 26 through 59 immediately upon entering the chamber, then hourly, each subject depressed a push button for an interval he estimated to be 10 seconds. This was repeated twice, then he estimated 30 seconds three times.

Beard-Wertheim Time Discrimination Test: In experiments 26 through 59 this test was performed immediately following the time estimation test. In experiments 26 through 45 the test was performed in a group setting while in the remainder of the experiments the subject was isolated either in the exposure chamber or in an audiometric booth to duplicate Beard's original conditions.

Reaction Time: The AAA driving simulator is a reaction-time testing device. A subject seated at a console is presented with one of three stimuli to which he must respond by turning his steering wheel right or left, or by removing his foot from the accelerator pedal and depressing the brake. Each of the stimuli are presented in a random but unvarying sequence. The time between stimulus and response for the 15 trials is automatically totaled by an accumulative timer.

Steadiness: The hand steadiness test is another AAA tester consisting of a gradually narrowing V-shaped vertical slot. A metal wand, held in one hand, is passed down the slot until one of the sides is touched. At this point, a light flashes, and the position of the wand is recorded. Scoring is tabulated by totalling the results of five trials.

Manual Dexterity: The Crawford collar and pin and the Crawford screw tests are measures of manual dexterity. Both tests give quite consistent results when learned and are easily administered. The Crawford collar and pin test requires the subjects to pick up a pin, about 5/8 of an inch long and approximately the same diameter as a paper clip, with forceps, place the pin upright in a hole, and ring the pin with a loosely fitting collar. The Crawford screw test requires the subject to pick up a short screw and drive it all the way through a threaded hole with a screwdriver. Both tests are scored on the basis of the number of tasks completed in three minutes.

Results of the driving simulator, hand steadiness, and the Crawford tests were all evaluated in a manner analogous to the time estimation tests. The latter tests, however, were only conducted once during each exposure at about seven hours into the exposure period. Results of these tests are also listed in table III.

Electroencephalogram and Evoked Response: The exposure chamber contained a copper screen-shielded cage with approximately 45 sq ft of floor area. Within this cage, an upholstered reclining chair with a back high enough to give support to the head and neck was used for EEG and evoked potential recording. Provocative procedures, including a short period of hyperventilation and photic driving, were included in the initial EEG examination.

### Analysis of Breath and Blood for Carbon Monoxide

Five milliliter aliquots of venous blood were collected in vacutainer tubes containing ethylenediaminetetraacetic acid. The blood was immediately analyzed by two methods. The hemoglobin concentration and the COHb percentage were determined directly in a CO-Oximeter (Instrumentation Laboratories, Inc.). The second analytical method consisted of measuring the CO liberated from the COHb moiety, using a GC equipped with a helium ionization detector (H. C. Dodd et al, unpublished data). Alveolar breath samples were analyzed directly by infrared spectroscopy (Stewart et al, 1965) and by the GC method (H. C. Dodd et al, unpublished data).

### RESULTS

#### Carbon Monoxide: 25, 50, and 100 ppm

No untoward subjective symptoms or objective signs of illness were noted during or in the 24-hour period following the exposures to 25, 50, and 100 ppm of CO. All of the clinical chemistries, including the repeat battery 16 hours following the 100 ppm or greater gas exposures, remained within the limits of normal. There was no detectable change from control values for the clinical tests listed under Experimental Procedure. Data for those tests judged to be most critical to the discussion are presented in tables III to V.

The only significant relationship was that of the Crawford collar and pin test versus CO concentration. In this case, the correlation coefficient,  $r$ , was significantly different from zero at the 0.05 level, indicating a decrease in the score with an increase in CO concentration. Two facts indicate that the apparent correlation was spurious. First, none of the paired t-tests for the pin and collar task even approached significance. Second, a similar task, the Crawford screw test, exhibited no correlation of score with CO concentrations.

For each concentration studied there was little individual variation in the absorption and excretion of CO, as reflected in the venous blood COHb concentration of the subjects. The COHb concentration was so predictable and reproducible for sedentary males from one experiment to the next that it could be expressed mathematically as a function of exposure time and concentration, as detailed in a companion article (Peterson and Stewart, 1970). The COHb data for the 50 and 100-ppm experiments are displayed in figure 2 and table II so that the concentration time relationship may be related to the performance data.

A predictable mathematical relationship was observed to exist between the post-exposure alveolar-breath CO concentration and the postexposure venous COHb concentration. This relationship is detailed in a second companion article (Peterson, 1970).

TABLE V  
REGRESSION ANALYSIS OF TIME ESTIMATION DATA

	n*	Correlation Coefficient	$r_{0.05}$
Concentration vs tone time estimate, < 3-hour exposure	21	-0.338	0.433
Concentration vs light time estimate, < 3-hour exposure	21	-0.128	0.433
Concentration vs tone time estimate, > 3-hour exposure	30	-0.247	0.361
Concentration vs light time estimate, > 3-hour exposure	30	-0.122	0.361
Stimulus duration vs tone time estimate, any exposure	51	-0.072	0.276
Stimulus duration vs light time estimate, any exposure	51	-0.191	0.276

\* Number of subjects participating in specific experiments.

### % CO Hgb During and Following CO Exposure

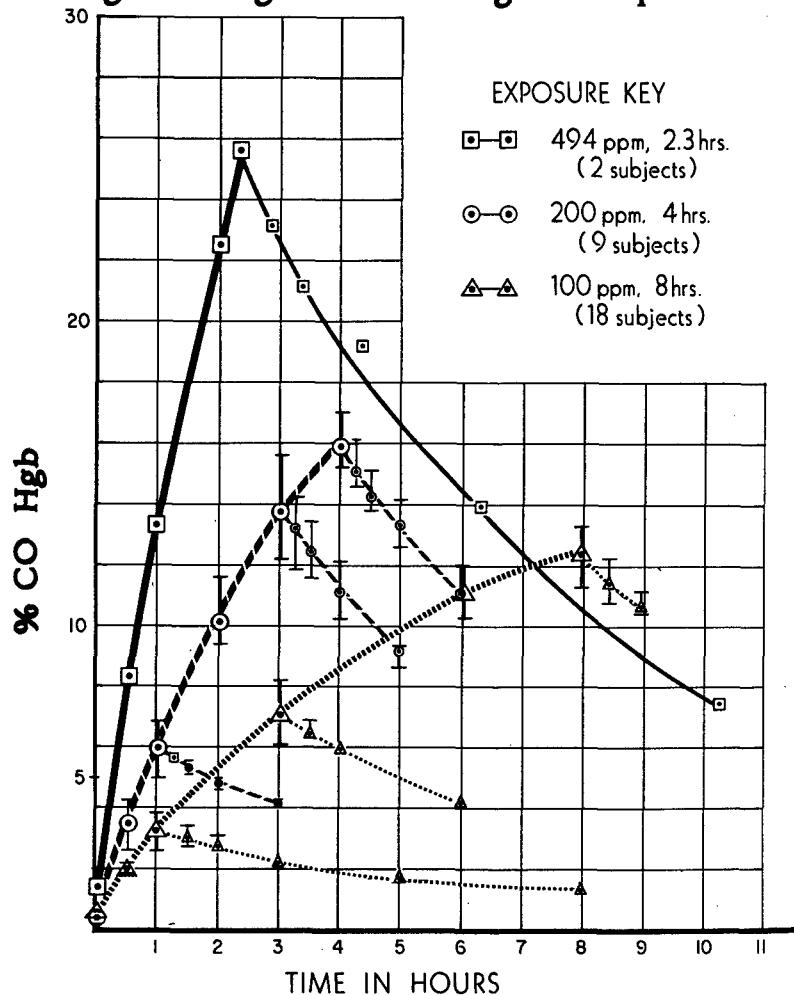


Figure 2. CARBON MONOXIDE ABSORPTION AND EXCRETION IN HEALTHY, SEDENTARY, NONSMOKING, WHITE MEN. (Reprinted with permission from the Archives of Environmental Health, 21:161, August, 1970).

Carbon Monoxide: 200 ppm, One to Four Hours

The three subjects exposed for four hours each reported that they had developed a "mild sinus" headache in the final hour. For one subject, this headache remained mild in intensity, subsiding completely in two hours. In the other two, headaches vanished during the first 30 minutes following exposure.

All of the clinical chemistries, including the repeat battery 16 hours postexposure, remained within the limits of normal. There was no detectable or statistical change from control values for the other clinical tests. The absorption and excretion of CO, as reflected in the venous blood COHb concentration of the subjects, are shown in figure 2.

There was no impairment noted in any of the performance tests. The Beard-Wertheim time discrimination test and the 10- and 30-second time estimation tests were initially performed in a group setting with twenty subjects divided into three separate groups for the random exposures to < 2, 50, 100, and 200 ppm. There was no detectable impairment in performance as a result of CO exposure. Using the paired t-test, the following t-values were calculated.

## BEARD-WERTHEIM TEST IN GROUP SETTING

After 2-hour exposure

Group <sub>&lt;2</sub>	vs.	Group <sub>50</sub>	t =	0.2700
Group <sub>&lt;2</sub>	vs.	Group <sub>100</sub>	t =	0.3724
Group <sub>&lt;2</sub>	vs.	Group <sub>200</sub>	t =	-0.7567

n = 14   Critical t<sub>.01</sub> = 2.779

## 10-SECOND TIME ESTIMATION TEST IN GROUP SETTING

After 2-hour exposure

Group $<2$	vs.	Group <sub>50</sub>	$t = 0.9647$
Group $<2$	vs.	Group <sub>100</sub>	$t = 0.1345$
Group $<2$	vs.	Group <sub>200</sub>	$t = -0.2098$

$n = 14$  Critical  $t_{.01} = 2.779$

## 30-SECOND TIME ESTIMATION TEST IN GROUP SETTING

After 2-hour exposure

Group $<2$	vs.	Group <sub>50</sub>	$t = 0.8945$
Group $<2$	vs.	Group <sub>100</sub>	$t = 0.3170$
Group $<2$	vs.	Group <sub>200</sub>	$t = 0.7786$

$n = 20$  Critical  $t_{.01} = 2.740$

Beard's subjects were isolated in an audiometric booth during their exposures and testing while our subjects were exposed and tested in a group situation. To investigate the significance of this difference four subjects were reexposed in a double blind experiment to ambient and 200 ppm CO concentrations while in an isolated situation - alone in the 20 x 20 x 8 foot exposure chamber or in an audiometric booth. Performance while isolated in the audiometric booth or in the large chamber was not statistically different from that as a member of a group.

Using the paired t-test, the following t values were calculated:

Group <sub><2</sub> vs. Isolated <sub><2</sub>      t = -1.135

Group <sub>200</sub> vs. Isolated <sub>200</sub>      t = 0.364

Alone <sub><2</sub> vs. Booth <sub><2</sub>      t = -0.000

Alone <sub>200</sub> vs. Booth <sub>200</sub>      t = -0.491

Isolated <sub><2</sub> vs. Booth <sub><2</sub>      t = -2.121

Isolated <sub>200</sub> vs. Booth <sub>200</sub>      t = 0.193

n = 4      Critical t<sub>.01</sub> = 5.48

#### Carbon Monoxide: 500 ppm

During the first exposure to 500 ppm of CO (experiment 22), one subject reported light-headedness after only 20 minutes of exposure. This was believed due to hyperventilation and persisted for 45 minutes. After one hour of exposure, both subjects were aware of a 10% increase in heart rate with the minimal exertion of walking to the blood sampling port. Ninety minutes into the exposure, the second subject noted the onset of mild frontal headache. Oxygen was administered by face mask immediately following the exposure, and within 10 minutes the second subject's headache was gone.

During the second exposure to 500 ppm of CO (experiment 23), the same subjects both developed mild frontal headaches after one hour of exposure. Minimal exertion caused a transient intensification of the pain. Both headaches remained mild during the first postexposure hour, then they intensified into excruciatingly severe occipitofrontal headaches, reaching a pain peak three and a half hours after exposure. These featured mild nausea, were not ameliorated by aspirin, and persisted for seven hours.

During the third exposure to 500 ppm of CO (experiment 24), the occurrence of mild frontal headaches again was noted after one hour of exposure. Immediately following the exposure, both subjects were placed in a hyperbaric chamber and administered oxygen at 3 ATA. The mild headaches were gone before the chamber was fully pressurized.

During experiment 25, the subjects were exposed to a constantly rising concentration of CO until a concentration of 1,000 ppm was reached after two hours. This peak concentration was then maintained for an additional 30 minutes. At the two-hour exposure mark, both subjects reported the presence of mild frontal headaches. Following the exposure, headaches became moderately severe over the first two postexposure hours. Six hours postexposure, the headaches were incapacitatingly severe and not ameliorated by aspirin. Twelve hours postexposure, after a night's sleep, the headaches were still noticeable.

All of the clinical chemistries, including the repeat-battery 16 hours postexposure, remained normal. Electrocardiograms taken at the conclusion of each experiment revealed no abnormalities. In experiments 23 and 24, a transient 10% increase in pulse rate was recorded every 15 minutes when the subjects would run in place for 15 seconds, but this was not different from the preexposure control values.

As the COHb saturation approached 20%, changes were observed in the VER. There was an increase in the amplitude of the 2-3-4 wave complex and a negative-going shift in the 5b and 5c segment. These changes increased as the saturation increased and promptly reverted to normal when COHb saturation fell below 15%.

In experiment 25, a series of control values for the two subjects for the Crawford collar and pin test were  $33.5 \pm$  and  $40 \pm 1$ . Five minutes before the end of the exposure, this test was repeated. Both subjects reported marked fatigue of hands and fingers while doing the test, and performance scores skidded dramatically to 29.5 and 32. One and one-half hours postexposure, the values were 33.5 and 38.5; hand fatigue was not noted.

The hand reaction time-time estimation test was also performed in experiment 25, 15 minutes before the conclusion of the exposure and again two hours postexposure. These data are presented in table VII and indicate a slight increase in reaction time two hours postexposure but no impairment of time estimation ability.

#### COMMENT

These experiments were conducted to obtain additional human toxicological information about CO over a range of carefully controlled concentrations. The low concentrations and the long durations of exposure which were chosen are those which may be encountered in urban and industrial settings. The brief exposures to high gas concentrations were included so that sufficiently high COHb saturations could be achieved which would result in untoward subjective responses and impairment of the subject's ability to perform the clinical tests.

TABLE VI  
MEAN PERCENT OF COHb SATURATION

	Experiment 22	Experiment 23	Experiment 24	Experiment 25
Pre-exposure	0.6	1.5	1.2	0.4
Exposure time	...	...	...	...
15 min	5.3	...	...	1.2
30 min	9.2	8.2	8.0	2.1
45 min	...	...	...	3.7
1 hr	16.6	13.7	13.1	6.4
1 $\frac{1}{4}$ hr	...	...	...	9.8
1 $\frac{1}{2}$ hr	...	18.4	18.1	14.3
1 $\frac{3}{4}$ hr	23.8	...	...	17.9
1.9 hr	24.8	...	...	...
2 hr	...	22.6	21.9	23.0
2 $\frac{1}{4}$ hr	...	25.4	...	28.1
2 $\frac{1}{2}$ hr	...	...	...	31.8
Post-exposure time	100% O <sub>2</sub> by mask		O <sub>2</sub> at 3 ATA	
10 min	23.0		13.2	
15 min	...		...	30.3
30 min	19.6	23.1	7.6	28.5
1 hr	...	21.1	...	25.6
1 $\frac{1}{4}$ hr	13.9	...	...	...
2 hr	...	19.3	...	20.2
2 $\frac{1}{4}$ hr	...	...	...	18.4
3 $\frac{1}{2}$ hr	...	...	...	15.6
4 hr	...	13.8	...	...
4 $\frac{1}{2}$ hr	...	...	...	13.3

TABLE VII  
HAND REACTION TIME - TIME ESTIMATION TEST

Experiment 25

Stimulus	Duration (sec)	Reaction Time (sec)	Time Estimate (E/D)*
<b>Pre-exposure</b>			
Tone	1	0.14	0.61
Tone	3	0.15	0.70
Tone	5	0.14	0.67
Light	1	0.17	0.68
Light	3	0.16	0.67
Light	5	0.16	0.64
<hr/>			
<b>Exposed 2.3 hours</b>			
Tone	1	0.18	0.58
Tone	3	0.19	0.68
Tone	5	0.15	0.68
Light	1	0.19	0.61
Light	3	0.17	0.61
Light	5	0.15	0.68
<hr/>			
<b>2 hour post-exposure</b>			
Tone	1	0.25	0.71
Tone	3	0.18	0.64
Tone	5	0.14	0.69
Light	1	0.21	0.56
Light	3	0.19	0.64
Light	5	0.14	0.66
<hr/>			

\* Actual duration.

The most important finding was that an eight hour exposure to 100 ppm of CO, resulting in a COHb saturation of 11% to 13%, produced no impairment of performance in the tests studied in this select, healthy group of volunteers. The tests chosen for investigation were those felt to be of practical significance in the performance of vocational endeavors and of automobile driving where significant impairment of visual or auditory acuity, coordination, reaction time, manual dexterity, or time estimation would be intolerable. The effect of exposures to 100 ppm of CO for eight hours on persons with preexisting cardiopulmonary diseases, on those consuming alcohol or central nervous system depressant drugs, and on the aged remains to be determined.

The subtlest effect of very small increases in COHb has been described by McFarland and his collaborators (1944). Visual brightness discrimination was impaired significantly when an increase of 4% COHb saturation had occurred. Conflicting reports are in the literature regarding the effects of small increments of COHb on flicker fusion frequency (Lilienthal and Fugitt, 1946; Vollmer, 1946). The significance of these subtle alterations in vision to those in various vocations remains to be defined. In our series of experiments, no significant impairment of those parameters of vision studied occurred as a result of eight hours of exposure to concentrations of CO as high as 100 ppm.

Schulte studied the effect on middle-aged firemen of exposure to 100 ppm of CO for varying periods of time (1963). The majority of his subjects were smokers. Included was a battery of psychomotor tests, and for some of these which tested cognitive abilities and choice discrimination, he reported variations in performance at COHb saturation concentrations below 5%. Reaction time, static steadiness, and muscle persistence were not altered by concentrations of COHb up to 20%. However, the very high COHb concentrations reported after exposures to only 100 ppm raises the question of reliable analytical techniques. Nonetheless, Schulte's observations regarding the adverse effect of CO on cognitive abilities and choice discrimination merit further investigation.

Beard and Wertheim (1967) have reported a distinct disturbance in the ability of healthy subjects to perceive differences in the duration of auditory stimuli following a 90-minute exposure to CO in concentrations as low as 50 ppm. (Carbon monoxide exposures of this magnitude for this duration would result in an increase of COHb saturation of approximately 2% above baseline). They further reported that decrement in performance increased dramatically with CO exposures of greater magnitude. We were unable to confirm these observations during exposures to CO in a group setting.

Our initial study to investigate the influence of the audiometric booth and isolation as a factor indicated that neither is a significant factor. While the "n" of four was small, it was large enough to detect the dramatic impairment in both time estimation and discrimination reported by Beard and Wertheim. This series is being enlarged to rule out the possibility of overlooking a CO effect much more subtle than that reported by Beard.

There were additional differences between the tests of Beard and our own. The difference of gravest concern was that Beard's tests were single blind studies, while ours were double blind. Beard and Wertheim's subjects were confined in a small audiometric booth which featured a black interior and no temperature or humidity control. Their chamber CO monitoring was done with a single non-dispersive infrared spectrometer in contrast to the multiple, independent monitoring systems here described. Beard and Wertheim report that they did not run CO standards from within their booth to their infrared instrument and that they were not able to analyze preexposure and post-exposure blood samples for COHb (1967). Without knowledge of subject COHb saturation, there is no second check on the magnitude of their CO exposures.

In our study, the ability of subjects to estimate time intervals of 1, 3, 5, 10, and 30 seconds was not impaired by CO exposures which resulted in COHb concentrations several fold higher than those which should have been encountered by Beard and Wertheim. The authors consider these time-estimation data of sufficient value that data from experiments 6-8, and 11-13 have been included in tables III to V. The difference between our findings and those of Beard and Wertheim merits resolution if the effect of CO absorption on time sense is to be known.

Those exposures which increased the COHb saturation to 20% or greater produced both subjective and objective evidence of CO intoxication. The first consistently present symptom of illness was the onset of a barely perceptible frontal headache occurring after the COHb saturation rose above 15% to 20%. An observation of clinical concern was that the severe headache of CO intoxication was a delayed phenomenon in the sedentary subject. It was apparent that exposure to a potentially lethal concentration of CO over a period of a few hours might occur without producing good warning symptomatology. It was also of interest to observe that the prompt administration of oxygen before the headache of CO became intense would quickly abolish all head pain.

As COHb saturation approached 20%, changes were observed in the VER. These changes became more marked as the COHb saturation neared 30%. The VER was the most sensitive objective indicator of CO effect.

The only time a manual dexterity test was performed when the subjects were grossly overexposed was in experiment 25, when the COHb saturation was 28%. Dramatic impairment of this function was observed. Following the exposure, but when the COHb saturation was approximately 22%, manual dexterity appeared normal. This suggests that significant impairment of manual dexterity could occur before a subject would otherwise be aware of CO intoxication.

In these experiments, the accuracy of the CO and COHb determinations is considered to be reliable because the analyses were performed by two independent methods. The breath CO was analyzed by infrared and by GC (H. C. Dodd et al, unpublished data) while the COHb was determined by the GC method and by the CO-Oximeter. The ability to confirm each blood and breath CO concentration during and following exposures to known CO concentrations provided the data with which to mathematically express the absorption and excretion of CO by these subjects. These regression equations are detailed in a companion article (Peterson and Stewart, 1970).

A second dividend from the accurate analyses of blood and breath was the opportunity to mathematically express the exponential relationship between venous blood and COHb concentration and alveolar breath CO concentration. This is discussed in detail in a second companion article (Peterson, 1970).

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## DISCUSSION

MR ADAMS (School of Aerospace Medicine): You made reference to cardiac output, now how did you measure this?

DR. STEWART (The Medical College of Wisconsin): We did this indirectly using the impedance cardiogram technique. We feel that this is a very reproducible, good way to measure cardiac output by a noninvasive method. It allows you to very nicely compare the individual with himself from setting to setting.

DR. ROBERTSON: You mentioned that females had a more rapid uptake and excretion of CO. Would you hypothesize why?

DR. STEWART: Yes, they breathe slightly differently than do the males. It is particularly obvious when we let them do a little exercise where we keep the exercise constant where the male might be a little more able to perform a given workload. A lighter female at the same workload breathes a little harder but we see then a more rapid uptake and a more rapid excretion.

DR. BEARD: Dr. Stewart, in your comparisons of performance, have you compared the subject against his own performance without carbon monoxide, or have all of the comparisons been based on group means?

DR. STEWART: We analyzed the data in several fashions. We have done it using group means, we have done it using the individual, using paired "T" tests, and in no instance have we found any CO effects.

DR. SALTZMAN (University of Cincinnati): From your work would you conclude, in an ordinary case where humans are exposed to fluctuating concentrations of carbon monoxide, that the only significant exposures of concern are the spikes, brief peaks, and I assume that this would be a matter of even a minute or two--would you regard this as a fair conclusion from your work?

DR. STEWART: I haven't gone that far in my thinking. I think the rate at which carboxyhemoglobin builds up in the blood stream may well be a very critical factor. I think that so far as the heart is concerned, it seems exquisitely sensitive to this change in rate of buildup. I think so far as effect upon central nervous system is concerned, that when all of the work has ultimately been completed, we will find there is a difference in performance at whatever level we begin to find CNS effects while one is ascending to equilibrium and once one gets at equilibrium. For example in our

manual dexterity tests, when we were at 30% saturation, I was one of the subjects and found it very difficult to use forceps and do the Crawford "collar and pin test", which is my one claim to fame, it is the one area in which I can outperform the medical students--they beat me in every other regard, arithmetic, reaction time--I guess I'm getting old and breaking down. I noted that one of the reasons I was having great difficulty was because every time I would move my hand my muscles would ache in hands and arms. So the manual dexterity was somewhat impaired probably because of anoxia. Now I didn't know what would happen if I were to be maintained at 30% saturation--how well I might adapt. I have a feeling that I would have got quite well, if my heart would permit it, and that I might then later be able to perform that test quite well, so I think that there is a rigorously acute exposure and the same type of exposure allowing one to work at equilibrium.

DR. HODGE: Thank you very much, Dr. Stewart.

DR. BEARD: May I ask one more question, Dr. Hodge?

DR. HODGE: Yes, Dr. Beard.

DR. BEARD: What nature of motivation was driving your subjects, was there anything beyond their inherent desire to do well?

DR. STEWART: In all the group settings, there was among the medical students that we chose, that same competitiveness that I think kind of characterizes the breed. In the individual setting we tried to simulate exactly what you have done and we gave them the same monetary motivation, \$2.50 an hour during exposure for us to get rid of that variable, but otherwise we tried to duplicate in our setting as nearly as possible what we had photographed when you had graciously hosted our visit to your facility.

## ACUTE TOXICITY OF CARBON MONOXIDE UNDER HYPERBARIC CONDITIONS

Charles S. Rose, Lieutenant, USNR, MSC

National Naval Medical Center  
Bethesda, Maryland

### INTRODUCTION

The increasing importance of manned exploration under the sea has necessitated the development of data pertaining to the toxicity of contaminants in confined spaces under hyperbaric conditions. In any discussion of the effects of contaminants under pressure, carbon monoxide (CO) generates more than routine interest because of its unique mode of action and its numerous sources of generation--including production by man himself. The importance of considering atmospheric contaminants and their effects under pressure was emphasized by the identification of carbon monoxide in the breathing atmosphere of Sealab II in concentrations as high as 30 ppm at the surface.

A wealth of information has accumulated regarding the effect of CO on living systems at normal atmospheric pressure. In addition, studies have been conducted at hypobaric conditions. There is, however, little if any data pertaining to the acute toxicity of CO in intact animals under hyperbaric conditions. Therefore, the toxicity of carbon monoxide was evaluated under conditions of elevated pressure to determine if a pressurized environment would result in an altered response of an animal to the gas.

The toxicity of oxygen at high partial pressures is well recognized. No untoward effects, however, are noted when the partial pressure of oxygen is maintained at approximately 160 mm Hg regardless of the overall pressure of the system. It was reasoned, therefore, that the toxicity of carbon monoxide should depend solely on the number of molecules presented to the alveoli, if the partial pressure of oxygen in the environment remained constant.

Two criteria were utilized to evaluate CO toxicity: (1) a comparison of the LC<sub>50</sub> of carbon monoxide at ambient atmospheric pressure and at elevated pressures, and (2) the percent saturation of blood hemoglobin with carbon monoxide (carboxyhemoglobin) in animals which died during the exposures.

Experimental Animals

The animals utilized in these studies consisted of male Sprague-Dawley-derived rats, male Swiss albino mice, and male Hartley-derived guinea pigs. Prior to exposure, the animals were maintained on the appropriate food and water ad libitum. No food or water was allowed during the exposure.

Exposure Equipment and Materials

Exposures at ambient atmospheric pressure were conducted in a 30 liter cylindrical plexiglass chamber. A predetermined amount of carbon monoxide was mixed with 7.1 liters per minute of laboratory air and introduced into the chamber. The system used is shown schematically in figure 1.

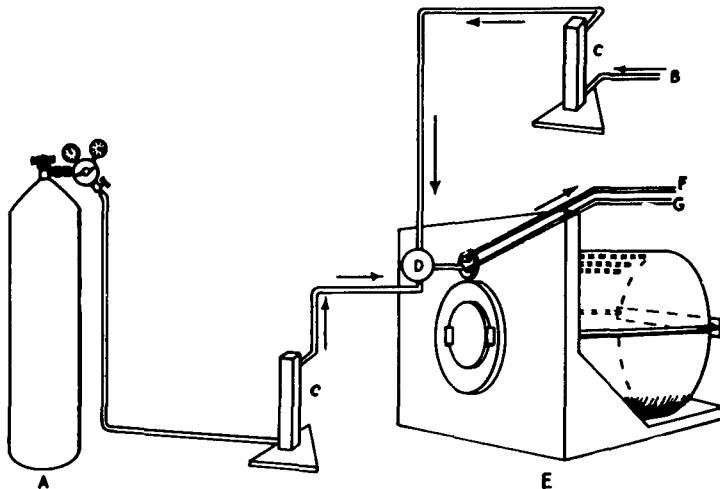


Figure 1. SCHEMA OF EXPOSURE SYSTEM USED AT 0 PSIG.  
 A, carbon monoxide cylinder (C. P. Grade) and regulator; B, house air for dilution; C, flowmeters; D, mixing flask; E, exposure chamber; F, exhaust line; G, sampling line.

The hyperbaric studies were conducted in an 8.6 liter Bethlehem Model 614 chamber rated for a maximum pressure of 150 psig at 70 F. For these exposures, certified premixed cylinders of carbon monoxide, oxygen, and helium were procured. Additional intermediate concentrations were mixed, as required, from the primary cylinders. During all runs, the partial pressure of oxygen was maintained between 140 and 160 mm of Hg by decreasing the oxygen from 21% (0 psig) to 7.6% (25 psig), 4.6% (50 psig), 3.3% (75 psig), and 2.6% (100 psig). In a similar manner, the various

concentrations of carbon monoxide in the gas mixtures introduced into the chamber for the LC<sub>50</sub> determinations had to be lowered concomitantly with the stepwise increases in pressure.

The pressure in the chamber was continuously monitored using a top mounted pressure gauge with a range of 0 to 100 psig. During exposures, chamber pressures were maintained within  $\pm 0.5$  psig of the desired pressures. The gaseous mixtures containing carbon monoxide were dynamically fed into the chamber through 1/4 inch flexible tubing and an exhaust flow rate of 4 to 5 liters per minute was maintained during the exposure period. Following all exposures, the chamber was decompressed with a mixture of 79% helium and 21% oxygen at a predetermined uniform rate depending on the experimental pressure. Rapid decompression was not used since at times one or more animals would not be visible through the chamber window at the termination of an exposure and an accurate death count would not have been obtained. The system used is presented diagrammatically in figure 2. The thermal variation of the chamber atmosphere as measured by a thermocouple, did not exceed  $\pm 2$  C of room temperature (23 C) during the animal exposure and decompression phases.

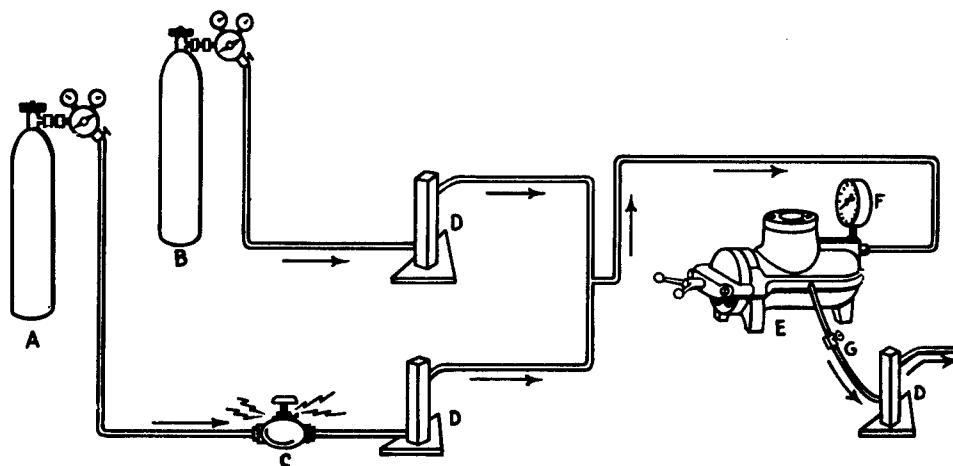


Figure 2. SCHEMA FOR HYPERBARIC EXPOSURES AT 25, 50, 75 AND 100 PSIG. A, cylinder containing decompression mixture of 79% helium, 21% oxygen with regulator; B, cylinder containing mixtures of carbon monoxide, oxygen, and helium with regulator; C, motorized valve; D, flowmeters; E, hyperbaric chamber; F, pressure gauge; G, exhaust flow regulator valve.

In both the 0 psig and hyperbaric studies, chamber loadings consisted of 4 rats, 4 guinea pigs, or 16 mice and all exposures were of 4 hour duration. A 4 hour exposure period was arbitrarily selected because of its general use in this and other laboratories in acute LC<sub>50</sub> studies with other materials at normal atmospheric pressure.

Immediately following the exposures at 0 psig or the decompression phase after the hyperbaric runs, cardiac blood samples were collected from the dead rats and guinea pigs and analyzed for carboxyhemoglobin concentration. The surviving animals were not observed further and were sacrificed.

### Analytical Techniques

In all exposures at normal atmospheric pressure, the actual concentration of CO was continuously monitored throughout the 4 hour period by a non-dispersive double beam infrared spectrophotometer set at a wavelength of 2160 cm<sup>-1</sup> and using a 5.65 liter variable pathlength gas cell. All laboratory mixed cylinders for the hyperbaric exposures were analyzed for carbon monoxide concentrations by the infrared spectrophotometer or by a gas chromatograph using a nickel oxide catalyst which reduced CO to methane. No monitoring during the run was considered necessary.

### Carboxyhemoglobin Method

Blood carboxyhemoglobin concentration was determined by a method based on the work of Stowe and Pelletier (1968) using a two-channel automatic chemical analyzer. A specimen of blood, with ethylenediaminetetraacetic acid as the anticoagulant, was split into two streams. Total hemoglobin was determined in one stream as cyanmethemoglobin at 550 m $\mu$ , and the carbon monoxide was released from hemoglobin in the other stream with 10% H<sub>2</sub>SO<sub>4</sub>. The gas phase was then removed with a trap and reacted with the silver salt of p-sulfaminobenzoic acid in alkaline solution. This resulted in a colloidal solution of silver which was measured spectrophotometrically at 420 m $\mu$ .

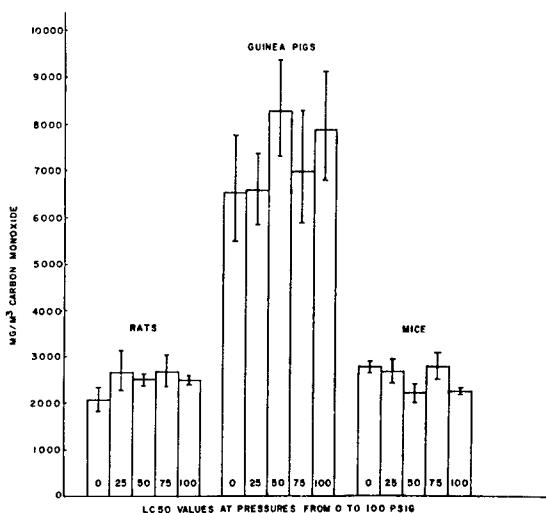
## RESULTS AND DISCUSSION

In general, all animals lost consciousness during the first 1 to 2 hours of carbon monoxide exposure. Table I presents the LC<sub>50</sub> values and their 95% confidence limits at pressures from 0 to 100 psig. As can be seen, there is very little variation in the absolute amount of carbon monoxide at the 50% mortality level in rats, guinea pigs and mice as the pressure is increased in stepwise fashion from 0 to 100 psig. Figure 3 is a pictorial representation of the data presented in table I. The small variation in the CO LC<sub>50</sub> values between pressure levels within a particular species was not considered to be biologically significant. It was also noted that guinea pigs were considerably less susceptible to CO intoxication at all pressures than were the rats and mice.

TABLE 1

LC50 Values for Carbon Monoxide  
at Pressures from 0-100 psig

psig	RATS CO LC50 (mg/m <sup>3</sup> )	GUINEA PIGS CO LC50 (mg/m <sup>3</sup> )	MICE CO LC50 (mg/m <sup>3</sup> )
0	2070 (1831-2341) <sup>a</sup>	6550 (5509-7788)	2800 (2679-2926)
25	2670 (2278-3129)	6600 (5888-7399)	2700 (2457-2967)
50	2500 (2372-2635)	8300 (7339-9387)	2230 (2046-2431)
75	2680 (2354-3060)	7000 (5902-8302)	2800 (2528-3101)
100	2500 (2385-2620)	7900 (6834-9132)	2270 (2202-2340)

<sup>a</sup> 95% confidence limitsFigure 3. LC<sub>50</sub> OF CARBON MONOXIDE IN RATS, GUINEA PIGS, AND MICE AT 0, 25, 50, 75 AND 100 PSIG.

This phenomenon correlates with the lower affinity constant of hemoglobin for CO in guinea pigs than in either rats or mice. The approximate ratios of the partial pressures of CO to O<sub>2</sub> at the LC<sub>50</sub> are given in table II as well as the mean carboxyhemoglobin values in rats and guinea pigs which died during the exposures at each pressure. Both the COHb levels producing death in rats and guinea pigs and the partial pressure gas ratios exhibited little variation regardless of the total pressure of the exposure environment.

TABLE 2

Partial Pressure Gas Ratios and Carboxyhemoglobin Percentages<sup>a</sup> for Rats and Guinea Pigs Exposed to Carbon Monoxide at Pressure

psig	RATS		GUINEA PIGS	
	pCO/pO <sub>2</sub> for LC <sub>50</sub>	% COHb Mean±S.D.	pCO/pO <sub>2</sub> for LC <sub>50</sub>	% COHb Mean±S.D.
0	0.009	57.5±6.9	0.029	77.6±10.4
25	0.012	62.1±15.8	0.029	71.5±19.8
50	0.011	60.7±5.7	0.036	67.5±16.1
75	0.012	58.8±8.7	0.031	67.6±12.9
100	0.012	64.9±9.8	0.036	65.0±8.3

<sup>a</sup> Values based on all concentrations of CO which resulted in animal deaths at a particular pressure

Berger et al (1964), using a carbon monoxide-air mixture, has shown in vitro that the equilibrium percentage of blood carboxyhemoglobin produced by a given concentration of CO was independent of the environmental pressure. More recently it has been demonstrated that the relative affinity constant of hemoglobin for carbon monoxide from both whole blood and prepared hemoglobin solutions was not significantly affected by elevated pressure or the inert gas component of the pressurized atmosphere (Rodkey et al, 1969). The carboxyhemoglobin values obtained from intact animals at death in the present studies support these findings.

Henderson and Haggard (1943) indicated that at equilibrium the distribution of hemoglobin between carbon monoxide and oxygen depended on the ratio of the partial pressures of CO to O<sub>2</sub> as well as the affinity of hemoglobin for these two components. Berger et al also suggested that the apparent toxicity of CO should be unaffected if the ratios of these two gases remained constant. The partial pressure gas ratios reported in table II support this hypothesis.

It is anticipated that many additional atmospheric contaminants will be encountered in manned exploration under the sea. The results reported here indicate that the toxicity of carbon monoxide is not altered by increases in ambient pressure up to approximately 8 ATA provided the partial pressure of oxygen in the atmosphere remains constant. Carbon monoxide, however, is unique in its mode of action and no attempts were made to evaluate any subjective effects, chronic effects, behavioral effects or measurements of decrements in performance during the exposures. Therefore, one should not generalize from data on carbon monoxide as to the toxicity of other materials under hyperbaric conditions.

This presentation is based on a paper published in the *Journal of Toxicology and Applied Pharmacology*, Vol. 17, p. 752-760, November 1970.

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## DISCUSSION

DR. MAC FARLAND (York University): The LC<sub>50</sub> values you gave are four hour LC<sub>50</sub>'s?

LIEUTENANT ROSE (National Naval Medical Center): Right. Four hour exposures.

DR. MAC FARLAND: Did you ever observe a death after the exposure was terminated?

LIEUTENANT ROSE: All surviving animals were sacrificed immediately following exposure.

DR. MAC FARLAND: That's not my question. Did you ever observe a death after the exposure was terminated?

LIEUTENANT ROSE: During the decompression one time, the decompression period after the CO was shut down, there was one death in the guinea pigs. That was not tabulated in the LC<sub>50</sub> data, but that was the only time.

DR. MAC FARLAND: Yes, you see the question in my mind is this, carbon monoxide is very unusual, it is easy to determine the LT<sub>50</sub> for carbon monoxide exposures, but the LC<sub>50</sub> is difficult because as soon as you terminate the exposure, essentially the animals now start to recover. From the curves we saw in the preceding paper, as soon as the exposure is terminated the animal begins to blow off carbon monoxide so that deaths postexposure are almost unknown and this seems to have been your experience. I have a feeling that the proper determination of the LC<sub>50</sub> with carbon monoxide for a stated exposure period, say four hours, would involve conducting exposures, the duration of which was longer than four hours in order to get the top points on the regression line above the LC<sub>50</sub>, but I'm not certain of this, but carbon monoxide is unique in this regard, it is an interesting problem. Thank you.

MR. HAUN (SysteMed Corporation): May I ask what were the numbers of animals in each group and the weights of the animals?

LIEUTENANT ROSE: The weights of the rats ranged between 225 and 300 grams; the guinea pigs between 300 and 350, and mice between 25 and 28 grams. I don't remember the exact figures for total number of animals utilized. For rats, it was approximately 120, guinea pigs approximately 80. For mice it was considerably more because we used 16 for exposures instead of four.

MR. HAUN: The numbers per group exposed were how many?

LIEUTENANT ROSE: Approximately 30 to 40 per group at any one pressure.

## THE EFFECT OF EXPOSURE TO LOW CONCENTRATIONS OF CARBON MONOXIDE

John Schulte, M. D.  
Ohio State University

We are about half way finished with a vast research project at Ohio State. In fact, one could describe it right now as a "half-vast" research project. It's apparent that there are a lot of questions in all our minds about the differences in our findings and I would like to just very briefly review my own findings on my studies and those with which I have been affiliated at Ohio State, and then discuss the possibility of explanations for these differences. I did a study on 50 firemen over a period of nine months; I studied each one of them four times under four different sets of exposures so that they had approximately 5, 10, 15 and 20% carboxyhemoglobin levels in them. It was an economy experiment so in some ways maybe it was better as far as the results were concerned in that the carboxyhemoglobin levels as found went the complete gamut of 0 to 20%, in fact one of them had 20.5%--that was the maximum. Based upon these studies, I did find that there was an increase in arithmetic mistakes. The arithmetic test I used was to take the columns of figures out of a table of random numbers and use five digits, five columns, and they merely added them up. There were some 60 arithmetic problems in the test. I measured them for time and number of errors. This was, of course, double-blind as all good studies are whenever possible. I knew the subject myself, no other computer knew what the answer was until we finally cranked in the COHb levels. I also found some changes, an increase in the number of errors and a slowing down of the individual in reading poetry that had been printed--I forgot what poem it was but it was one of Shakespeare's sonnets, in which the subject had to underline all the plural nouns, and I counted the number of mistakes they made in this, also the number of mistakes they made in underlining the words that weren't nouns, just because they happened to end in an "s"--things of that nature. We analyzed it in addition for the unusual plurals like mice, meece, and so on, and separated that out and this didn't change the results at all. So we did find some changes in our study, which have been denied by other studies. Recently at Ohio State we did a field study using automobiles. We have two Plymouths that are worth approximately \$25,000 apiece, and they are completely instrumented for the things we have wanted to do. The lead car has a yo-yo system to the second car and the driver of the second car who has been exposed to some level of something to be evaluated is measured in his activities in responses to the first car. There is, of course, a second driver in the second car with dual controls in case we went a little awry. These cars have been used on our own oval track. They have also been used out on I-70 up to 70 miles per hour. The tracking procedures that have been used consist of following the lead car and, at predetermined times, the following

driver must either decrease the distance or increase the distance between the two cars or maintain the exact distance from the lead car, speeding up, slowing down, or remaining constant, and this has been timed out carefully as to which he is doing. The sequence has been changed on each test run. The driver of the second car is recorded when he eases off the accelerator pedal and when he puts on the brake in response to seeing the brake lights on the front car. There were four runs per subject made on that preliminary study: one with the subject at no exposure to carbon monoxide; one with the carboxyhemoglobin level at roughly 5%; one at roughly 10%, and one at roughly 15%. There were only three subjects--this is not a very large sample. The indications are, however, that somewhere between 5 and 10% there is an increase in judgmental error, especially on maintaining distance and closing. They don't seem to realize they're closing. That's even more important than maintaining the distance, actually. These are the only two studies that I have to report on of any interest to you at the moment. We are going to continue the road test studies with a larger test sample and under other circumstances and combine this with laboratory tests to see whether there is any prediction between laboratory findings and findings out in the field.

What I would like to do at the present time is philosophize a bit about why we have all these differences in our results. None of us had an ax to grind and if we've made a mistake in our study, it has been an honest mistake. Most of us have so many colleagues working with us that the chances of a mistake are almost nil. So it must be something else. One of the things which I've felt has been a very important consideration in this, and may account for some of the differences, is that in all the earlier work, and this includes my own study, the subjects were banged pretty hard with a high concentration of carbon monoxide for a short period of time and then maintained at that level or immediately examined and I wonder whether there isn't a big difference between getting to 15% carboxyhemoglobin if you do it by breathing 100 parts per million for roughly 12 hours, 250 parts per million for 4 hours, or 500 parts per million for an hour and a half. And I wonder seriously whether this might not account for the differences in our findings. A second complicating factor is the possibility of combined stresses. Some of us did not have environmental chambers at the time we did our studies or may not even have them now and may have had to resort to gas masks, things of this nature. Therefore, we have added a second stress or maybe more stresses depending upon the location of our laboratory. As Dr. Stewart pointed out, he has a country club to do his studies in and this may be a very important factor that we're adding two, three, maybe five stresses, none of which by themselves would produce anything but when all put together definitely produce effects. The third situation is one to which I have never received a satisfactory answer and I have asked a number of psychologists. We are talking about cognitive levels, judgment, thinking, ability to think, and ability to respond, and are we really always measuring the same thing? Or are we measuring gradations between true high level thinking and conditioned reflex? A study done several years ago in Russia, Pavlov's dog experiment, indicated that taking new dogs who have never been trained and exposing them to carbon monoxide to develop levels of carboxyhemoglobin between 5 and 15% definitely lengthens the time required for that dog to develop a conditioned reflex. Now when you take a dog who has a conditioned reflex and try to knock it out of him, the only reason you knocked it out of him is he is half dead--you have to get between 70 and 80% carboxyhemoglobin

before they'll stop a previously established reflex. We talk glibly about arithmetic, how much of this is conditioned reflex. Most of us in this room are old enough that we took our arithmetic in grade school before modern math and we memorized the 12 times tables. Aren't they really reflexive? Everybody knows  $9 \times 9$ , off hand. You don't have to think about it. The same thing is true with adding and subtracting. The gimmicks some of us know have become reflexive, adding by 10, subtracting by one's--the rule of nine's, the rule of seven's, so on. This sort of thing may be a factor and in one case one man is really measuring cognitive thinking and another man doing a similar type test may really be measuring reflexes and therefore not get a change. There is one last problem in this area that may have caused some of the differences. Unfortunately no one can go back and prove it, and if it were known to be a fact it would immediately knock the poor researcher's results right straight in the head, and this is more a warning for future research than it is for anything that has happened to date. The question is how old was your carbon monoxide before you started to use it? Carbon monoxide has a nasty habit of forming carbonyls and if you have a tank full of that stuff that has been lying around for a while you may have some mighty toxic metal carbonyls in there that you really measured the results on rather than carbon monoxide. These are the three big factors, the big factors that I can see. There may be many others that could help account for this. I would welcome any suggestions or other possible explanations as to why we do not get the same results when apparently we did the same type of work under the same types of conditions, so if anyone has any suggestions, by all means say so.

I would finalize by warning of one other situation that does exist and that is in the literature, there is so much discussion of a paper starting out talking about automobile exhaust and ultimately concluding that this is carbon monoxide and the same thing with cigarette smoking. Well, I smoke cigarettes--they taste a heck of a lot different from an exhaust pipe, I assure you. The conclusions blamed on carbon monoxide as a result of exhaust pipes or cigarettes are very questionable. The results, the findings, are undeniable. It is the conclusions that you have to be on the lookout for.

## DISCUSSION

DR. SALTZMAN: I would just like to comment on this iron carbonyl problem. This has been studied by chemists, and since this is a reaction in a high pressure cylinder containing carbon monoxide, you can get an appreciable reaction with the steel walls. However, if there is any oxygen present in the cylinder this entire reaction is avoided because iron carbonyl is oxidized to iron oxide. So for those investigators who insist on using old mixtures, as long as it is a mixture containing some oxygen, they should not have any trouble. But if it is pure carbon monoxide it really is quite a serious consideration.

DR. BEARD: There are described some rather simple tests for the presence of iron carbonyl. That is simply burning a flame and the flame color is changed by the presence of the gas and then using a glass pipette as a burner there is a critical temperature area at which the iron will be deposited, at least so I read. We have made a practice of testing our cylinder gas in this way and have never found any iron carbonyl present. This we did not do in our earliest experiments, however, but never subsequently have we found any iron carbonyl and as a safeguard we make a practice of running the gas through activated carbon before running it into the exposure chamber.

DR. HODGE: Have you found, Dr. Schulte, any evidence of carbonyl in any of your samples?

DR. SCHULTE (The Ohio State University): Yes, I did on one occasion and unfortunately it was after I had done my study. It was not in my own gas samples but was at the experimental diving unit where we did have a few bottles of carbon monoxide in air. These were not merely weeks old, these were years old, however, and there was some iron carbonyl in them at that time.

DR. STEWART: I think regardless of the freshness of the cylinder, one has to assume that other toxic compounds could be present. Iron carbonyl needs to be routinely analyzed for and I think that the safeguard of running the gas when one is introducing it directly by mask to an individual through activated carbon is very essential. But in the same vein, another thing that is quite critical, I think in all these experiments, is to know what else is in the atmosphere besides carbon monoxide. What is the level of  $\text{SO}_2$ , the oxides of nitrogen, and the other compounds. One really has to look at these because in some of the older work there could have been combinations of compounds giving effects which were at that time maybe erroneously attributed to carbon monoxide.

DR. SCHULTE: That's very true.

MR. VERNOT (SysteMed Corporation): I'll just comment that in all the work we have done, both human and animal, we have analyzed every tank of carbon monoxide that we used for iron carbonyl and found the levels, if present, to be so low that they wouldn't even approach the TLV's listed in the literature.

## BEHAVIORAL RESPONSES TO SMALL DOSES OF CARBON MONOXIDE

Rodney R. Beard, M. D.  
and  
Netta Grandstaff, Ph. D.

Stanford University  
Stanford, California

The application of behavioral or performance measures to the evaluation of toxicity is fraught with complexities, as I shall demonstrate.

In January, Dr. Grandstaff and I prepared a review of studies on the effects of small doses of CO on cerebral function. This was presented at a symposium on CO sponsored by the New York Academy of Sciences. At that meeting, Dr. Mikulka reported observations on a tracking test made here at Wright-Patterson Air Force Base in Dr. Thomas' laboratory, which indicated much lower susceptibility to the effects of CO than had been assumed on the basis of work in our laboratory and the earlier report by John Schulte. At the same meeting, Dr. Rockwell reported aberrations of performance in operation of an automobile with COHb concentrations of the order of 10%; he had not tested lower concentrations. Since then, in August, Stewart and his colleagues have published a report that exposures producing COHb concentrations in excess of 15% to 20% resulted in delayed headaches, changes in the visual evoked response, and impaired manual dexterity. They found no impairment of a test of time estimation, nor of reaction time, nor of hand steadiness. The report indicates that mean values for groups of subjects were compared, rather than paired values for individual subjects, which might be a more sensitive test of variation of performance. In addition, Dr. Back and Dr. Thomas have informed me of their observations of great resistance to effects of CO on behavioral tests in monkeys, and reported them informally to a meeting of the BRC Toxicology Committee in May.

In view of these findings, we have reviewed some data of our earlier work, and have repeated the time-discrimination test, using a visual stimulus in place of the auditory stimulus formerly used. We have also carried out a problem-solving study which I shall report.

First, to review our earlier work: After experiments with rats on various operant behavior schedules indicated that timing behavior was readily affected in a dose related way, we set up experiments with healthy young human subjects. They were exposed in a commercial audiometer booth from which the air was continuously sampled and passed

through a non-dispersive infrared analyzer. To expose the subjects to CO, gas from a pressure bottle was metered by hand into the air inlet of the continuously ventilated chamber. A continuous recording of the concentration was made, and the flow of CO was adjusted to maintain the desired concentration. This has been the exposure pattern for all the experiments described.

In all experiments, the first 30 minutes in the chamber was spent in room air flowing at about 100 ft<sup>3</sup> per minute. The CO, if used, was quickly raised to the desired concentration, and held there for a predetermined time. After the CO was cut off, the concentration subsided in about 12 minutes or less. Most of the experiments have been "single blind", with the subject uninformed as to the presence or absence of CO. Subjects have been regularly asked if they thought CO might be present, and showed no ability to detect it.

In test 1, the subject's task was to judge whether the second of a pair of tones was shorter, longer, or the same as the first; the first tone was one second in duration, and the second could differ by 125 to 325 milliseconds. Scoring was the percent of correct responses in blocks of twenty-five trials; two trial blocks were run, taking about 8 minutes, then the subject rested for 12 minutes.

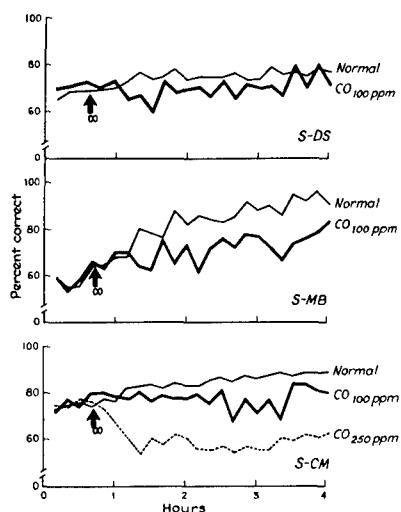


Figure 1. MEAN PERCENT CORRECT RESPONSES, FOR SUCCESSIVE 25-TRIAL BLOCKS, DURING AUDITORY-DURATION DISCRIMINATION. Data are from individual four-hour sessions for each of three subjects; arrows indicate gas exposure onset during CO sessions. "Normal" indicates a non-CO session.

As shown in figure 1, in the early tests, the subjects showed progressively better performance during a four-hour test session, and it was only by comparing specified trial blocks from days with CO and days without that a difference could be shown. Later, the subjects behaved quite stably. We cut down the length of the sessions to three hours, with 90 minutes of exposure to CO. Figure 2 shows the mean percent correct responses during minutes 30 to 90 of a two-hour exposure, plotted against various CO concentrations. This illustration is for a set of seven subjects. Similar results were obtained with eighteen others.

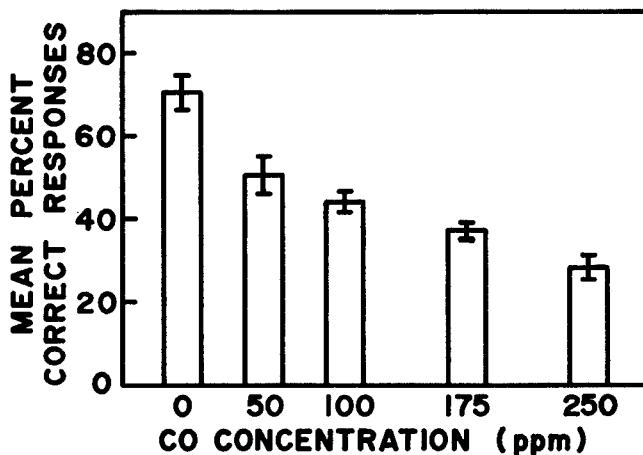


Figure 2. MEAN PERCENT CORRECT RESPONSES,  $\pm 1$  S. D., IN DISCRIMINATION OF TIME INTERVALS ABOUT ONE SECOND, DURING MINUTES 30-90 OF EXPOSURE TO INDICATED CO CONCENTRATIONS.

Figure 3 shows the time required to induce a significant change of performance.

We compared each sequential trial block with CO at a specified concentration against the same sequential block without CO; the average of the time elapsed before the proportion of correct responses fell 2 standard deviations below the control average is plotted against concentrations of CO.

We also tested another time-judgment function, the estimation of a time interval in the absence of any external clues to the passage of time. Figure 4 shows mean judgments of a thirty second interval, as influenced by CO. There is clearly a dose-related impairment shown.

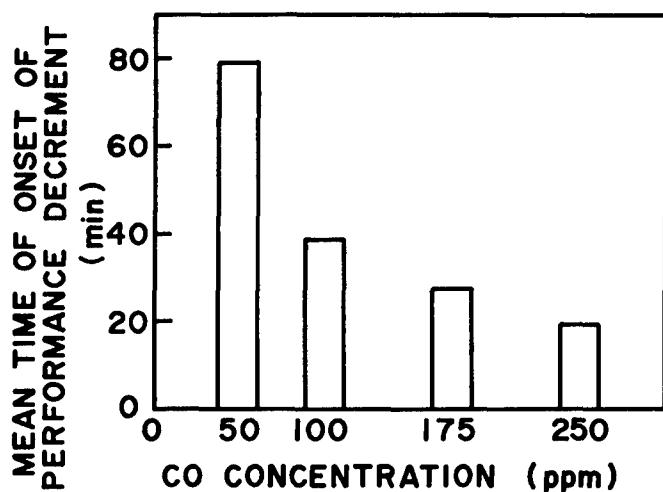


Figure 3. TIME AFTER INITIATION OF EXPOSURE TO INDICATED CO CONCENTRATION AT WHICH PROPORTION OF CORRECT JUDGMENTS OF TIME INTERVALS ABOUT ONE SECOND FELL 2 S. D. s BELOW MEAN PERFORMANCE WITHOUT CO.

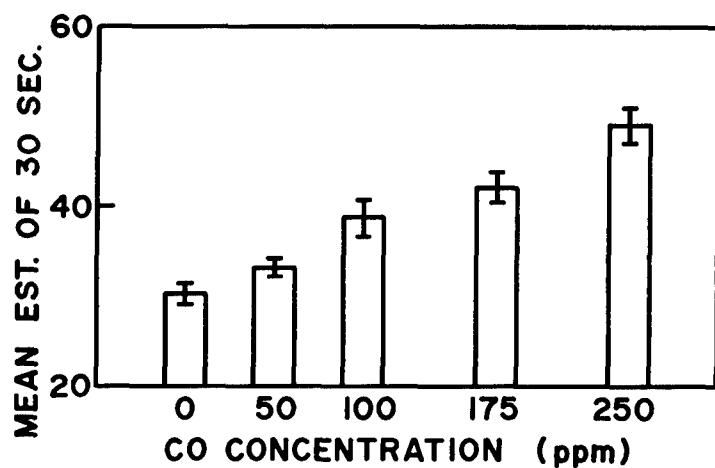


Figure 4. MEAN JUDGMENTS OF 30-SECOND TIME INTERVALS,  $\pm 1$ . S. D., AS A FUNCTION OF CO CONCENTRATION.

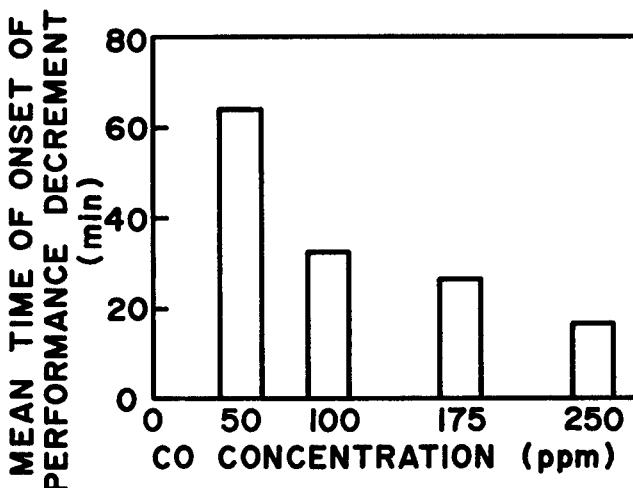


Figure 5. TIME AFTER INITIAL EXPOSURE TO CO THAT JUDGMENT OF 30-SECOND DURATIONS DEVIATED 2 S. D. S FROM THE MEAN JUDGMENT WITHOUT CO.

Figure 5 shows the exposure time required to cause a 2 standard deviation deficit in the presence of several CO concentrations, showing a significant decrement after less than 80 minutes at 50 ppm.

Results of a similar experiment, in which the subject was asked to press a thumb-switch at regular 10-second intervals are shown in figure 6. There is only a suggestive trend which was not statistically significant.

We next conducted a series of experiments on visual function. Figure 7 illustrates changes in the threshold for brightness discrimination at a low intensity, as affected by CO. As shown in figure 8, visual acuity, as measured by a variable grating, was similarly affected.

We have two experiments just completed, on which I can give you a preliminary report. First, we have worked for a long time toward a problem-solving type of test, in which there would be successive reinforcements for correct choices in a contingency situation.

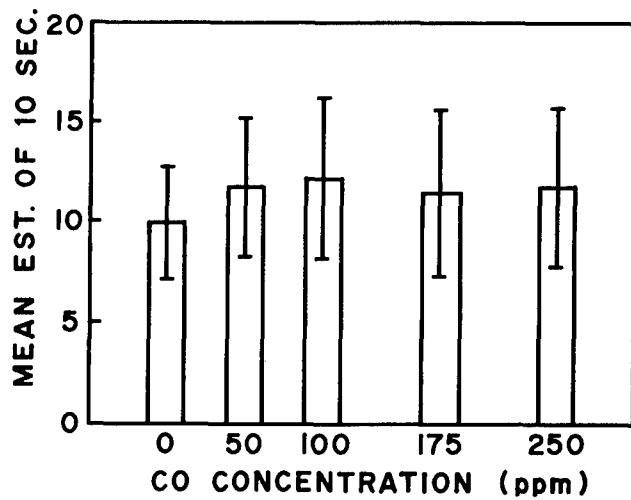


Figure 6. MEAN ESTIMATES OF 10 SECONDS,  $\pm 1$  S. D. OBSERVED DURING MINUTES 30-90 OF EXPOSURE TO INDICATED CO CONCENTRATIONS.

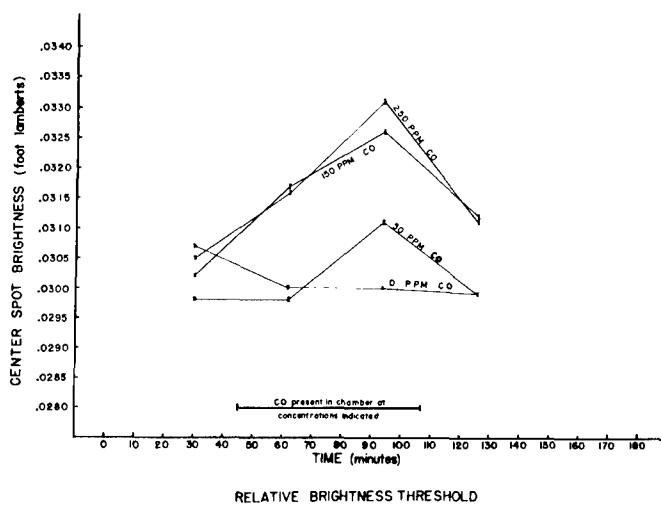


Figure 7. RELATIVE BRIGHTNESS THRESHOLD INCREASES AS A FUNCTION OF CO CONCENTRATION AND DURATION OF EXPOSURE.

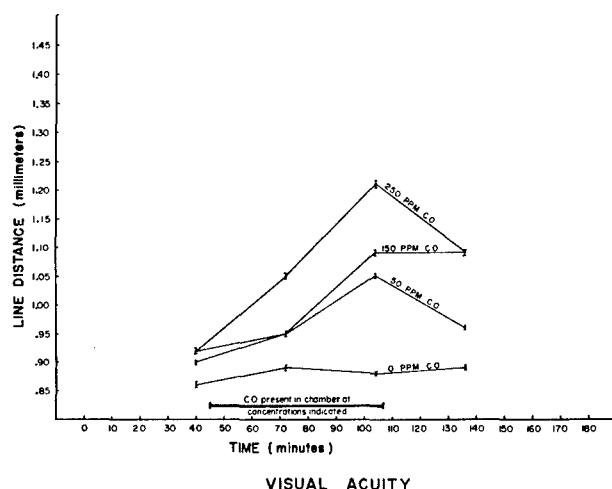


Figure 8. VERNIER VISUAL ACUITY DECREMENTS AS A FUNCTION OF CO CONCENTRATION AND DURATION OF EXPOSURE.

#### Display Experiment

Previous reports by several authors have emphasized that learned behavior patterns are more vulnerable to disruption by drugs when there is an increase in the complexity of the discriminative stimuli that control a given response or sequence of responses. We undertook what we refer to as the Display Experiment to determine the effects of low concentrations of CO on a series of complex sequential problems as well as to establish baseline data for further research on relative cognitive tasks.

The subjects were four young adult male and female students at Stanford University. They were all volunteers and received \$2.50/hour for participation in the testing. They received a small bonus for correct responses and a larger penalty for errors.

As in all previous experiments in this laboratory, each subject was required to fill out a questionnaire before and after each session. In this questionnaire the subject indicated his state of alertness, well-being, etc. In addition, each subject filled out a modified version of a standard medical history form. All appeared to be in good health.

During testing the subject was seated before a 5-inch oscilloscope screen which was located on one wall of the chamber. The subject was required to determine (by trial-and-error and remember a sequence of 15 different symbols from a 4 x 4 matrix of geometric symbols displayed on the oscilloscope screen (see figure 9). (All displays were generated and controlled by the PDP-8 computer). The subject was presented a series of tests (on each daily testing session) in which he had to learn a different sequence of 15 symbols for each test. The order of the sequence was predetermined

and randomized by the computer after each test. The subject made his response by touching a computer controlled light pen to his selected symbol. We used error scores and duration of response latency as our performance measures. These were automatically calculated and analyzed by the computer, and since response time is usually longer with various drugs, we predicted an increase in response latency with CO.

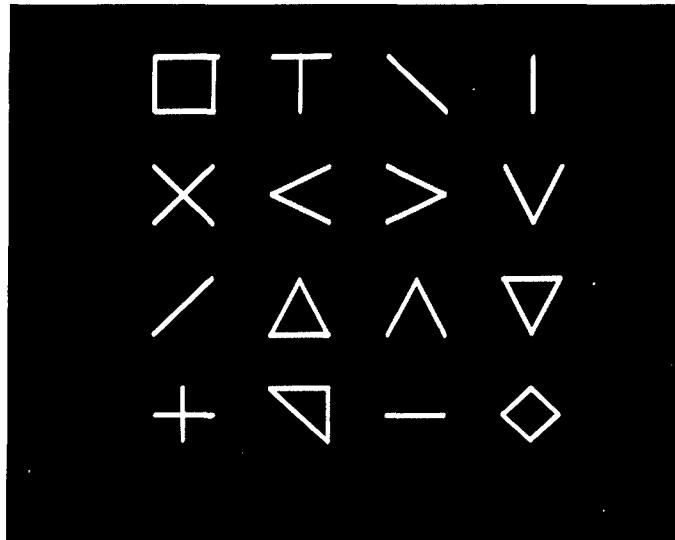


Figure 9. THE SIXTEEN GEOMETRIC SYMBOLS  
USED IN THE DISPLAY EXPERIMENT.

A single test consisted of 3 parts. During the first part, the subject determined the "correct" sequence of 15 symbols which he was then required to repeat twice (parts 2 and 3) in the correct serial order. In parts 2 and 3 if he chose the wrong symbol, omitted a symbol or added an extra symbol, this constituted an error. During part 1 the subject was, by necessity, given feedback on whether his response was correct or incorrect, but during parts 2 and 3 there was no knowledge of results given. Each test session was  $2\frac{1}{2}$  hours duration. The CO went on after 30 minutes, remained on for 90 minutes, and was off for the remainder of the session for all days of testing when CO was given. The subjects completed an average of about 12 single tests on any one day of testing (a session). To date, we have tested only the effect of 250 ppm of CO. There were four sessions with CO, three without.

As shown in figure 10a, the overall response latency did not appear to be altered reliably when CO was given. However, we decided to look at the response latencies only after correct responses during part 1 of each test. This is a time when the subject would normally be storing the necessary information for recall required for the second and third part of a given test. These response latencies became slightly longer in two out of the three subjects, as shown in figure 10b.

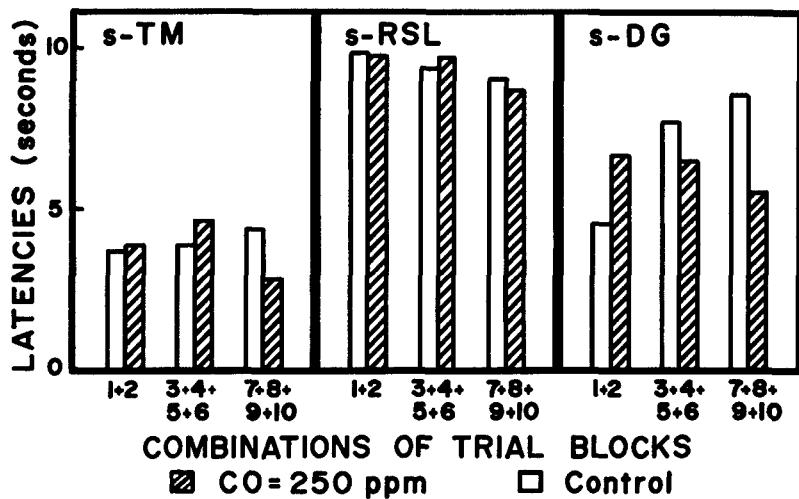


Figure 10a. RESPONSE LATENCIES FOR INDIVIDUAL SUBJECTS FOLLOWING CORRECT RESPONSES ON THE DISPLAY TEST FOR 0 PPM AND 250 PPM CONCENTRATION OF CO.

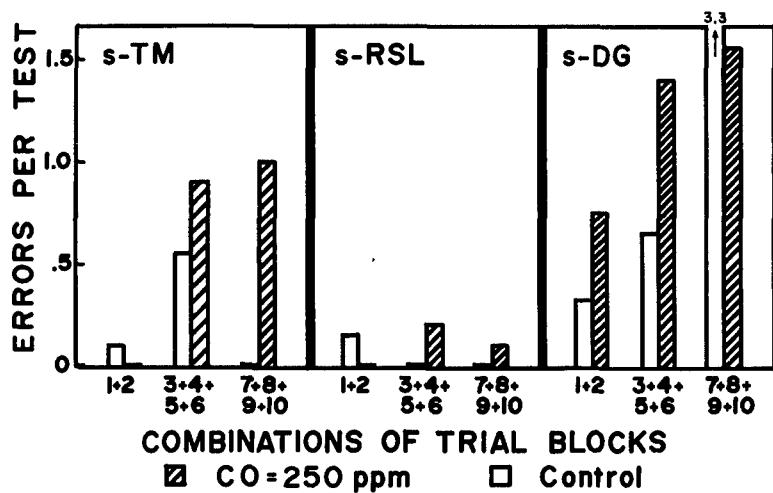


Figure 10b. MEAN ERROR SCORES FOR PARTS 2 AND 3 OF DISPLAY AT 0 PPM AND 250 PPM CONCENTRATION OF CO.

The performance errors were evaluated by comparing the average number of errors made during the period when CO was present with the same period of testing when gas was not given. The results show that when errors are averaged over all subjects, when gas is not present, the error rate is .47/test while during the same period with gas, the rate is .80/test. This indicates that with exposure to CO the error rate almost doubles (49%). The maximum number of errors possible per test is 30. These results certainly warrant further verification and investigation, so we plan to test more subjects on the Display task.

We have attempted a replication of our first experiment on temporal discrimination. In every respect we can recognize, the test circumstances differ in only four relevant ways, a) the test chamber was now in a temperature-controlled room, and b) the stimulus was now a visual signal instead of an aural signal; c) the later experiment was run in double-blind fashion; d) stimulus presentation, response recording and data analysis were now controlled and completed by on-line computer in our laboratory, instead of being stored on punched paper tape, translated to cards, and analyzed by the University computation center.

The subjects were four healthy, young adult students ranging in age from 18 to 26 years. They were paid \$2.50/hour for their volunteer participation in the experiment.

During the testing period the subject was seated in the chamber with a 3 x 3 inch panelescent light source, approximately two feet in front of his eyes. The rise and fall time of the light was less than 10 msec. All testing was computer controlled with a detailed print-out of the subject's performance, including an analysis of the data. We looked at error scores and response latencies as performance measures.

The duration of each test session was three hours with CO being present for a total of 90 minutes on those days when gas was given. The gas went on immediately following the second test (30 minutes after the onset of testing) and off immediately following the sixth test (90 minutes later). Testing began when a warning tone came on as a signal to the subject that he should position himself in front of the light panel and be prepared to respond. Fifteen seconds after the onset of the warning tone, the first pair of lights was presented. The subject's task was to judge whether the second of the pair was shorter, identical or longer than the first, and as soon as he reached a decision, he was to depress the appropriate response button. The first light persisted for one second. The duration of the second light varied from the first by 125 msec to 325 msec. There were 50 pairs of lights which followed each other at intervals of about 8 seconds for a total period of 8 minutes. This was followed by a rest period during which the subject could read, rest or study. At the end of the 12-minute rest period, the warning tone sounded and the next run of tests started. The same procedure was used throughout the testing session for a total of 10 runs for each test day.

Figure 11 shows the overall results for this experiment. No change is apparent for any concentration up to 250 ppm.

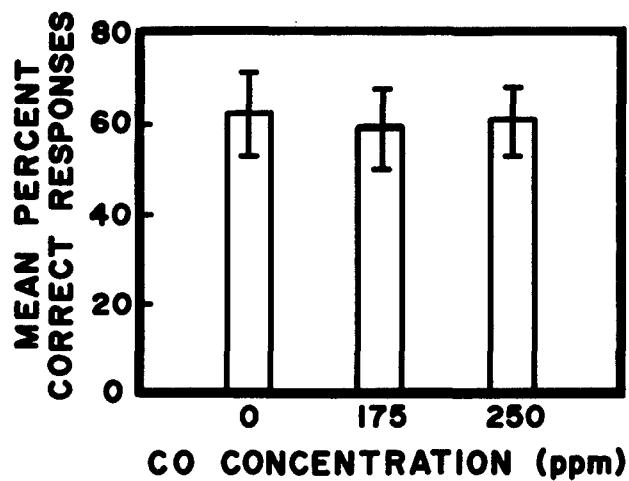


Figure 11. MEAN PERCENT CORRECT RESPONSES, IN DISCRIMINATION OF TIME INTERVALS ABOUT 1 SECOND USING A VISUAL STIMULUS DURING 30-90 MINUTES EXPOSURE TO INDICATED CO CONCENTRATIONS.

As shown in figure 12, looking at individual records, we see that subject DG shows an appreciable effect; the other three do not.

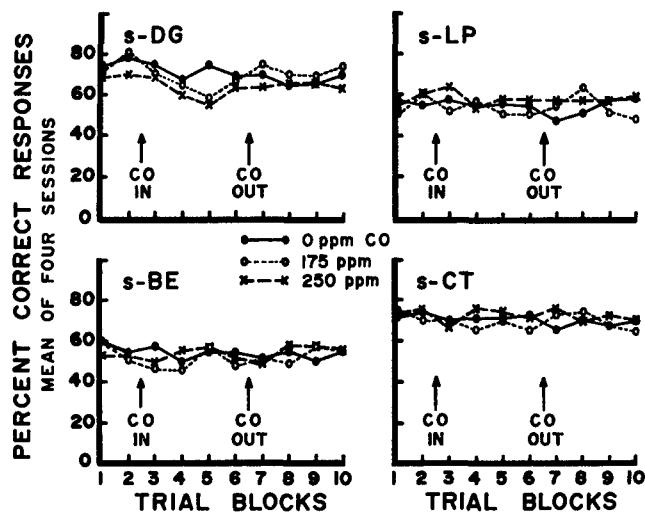


Figure 12. PERCENT CORRECT RESPONSES IN DISCRIMINATION ABOUT 1 SECOND USING A VISUAL STIMULUS DURING MINUTES 30-90 AT INDICATED CO CONCENTRATIONS FOR INDIVIDUAL SUBJECTS.

As a contrast, figure 13 shows the kind of record obtained in the earlier experiment.

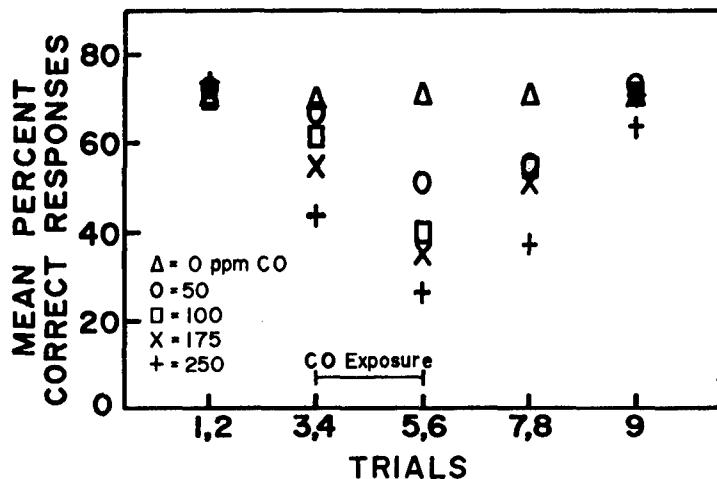


Figure 13. PERCENT CORRECT RESPONSES IN DISCRIMINATION ABOUT ONE SECOND WITH AN AUDITORY STIMULUS FOR ONE SUBJECT (TEST 1, EXPERIMENT 2).

Taking all of the results on this test for the same subject, DG, we can compare combinations of trial blocks, as shown in figure 14. For each concentration we have shown the summed results of the two preliminary blocks, then the four blocks while CO exposure was progressing during a 90-minute time span, followed by the three-blocks after CO was turned off. More sophisticated combinations, taking into account the small dose of CO in block 3 and the persistence of CO in the chamber during block 7, have been done, but show nothing more. It will be seen that only with an exposure to 250 ppm is there a significant decrement in performance, and this only in one subject among four.

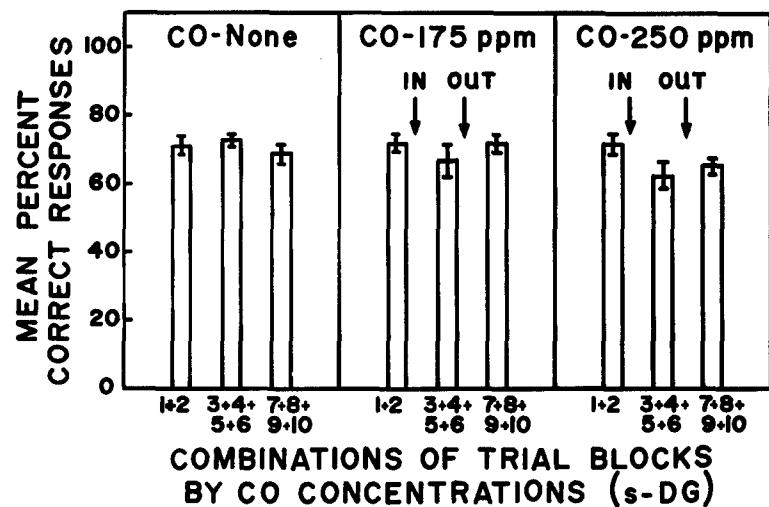


Figure 14. MEAN PERCENT CORRECT RESPONSES FOR DISCRIMINATION ABOUT 1 SECOND USING A VISUAL STIMULUS FOR SUBJECT D. G.

THE EFFECTS OF CARBON MONOXIDE ON CORONARY  
HEMODYNAMICS AND LEFT VENTRICULAR FUNCTION  
IN THE CONSCIOUS DOG

Jim D. Adams

Howard H. Erickson, Major, USAF, VC.  
Hubert L. Stone, Ph. D.

USAF School of Aerospace Medicine  
Brooks Air Force Base, Texas

INTRODUCTION

The physiologic effects associated with the exposure to high levels of carbon monoxide have been documented. However, little and conflicting data is available concerning the exposure to subclinical levels of carboxyhemoglobin (COHb). Carbon monoxide (CO) affects the central nervous system in some undefined manner and interferes with the transport and utilization of oxygen ( $O_2$ ). Hemoglobin and porphyrin compounds in general have a very high affinity for CO as compared to oxygen. The competition between CO and  $O_2$  for hemoglobin is manifested in the pulmonary capillaries. The results of an elevated inspired  $P_{CO}$  increases the percentage of COHb in the arterial blood. This is accompanied by a corresponding decrease in the oxygen saturation. Thus the arterial oxygen available for transport to the tissues by the cardiovascular system is reduced. Tissue hypoxia results even though the arterial  $P_{O_2}$  is not significantly lower than normal.

Hypoxia has the greatest effect on tissue beds that extract high percentages of the available oxygen, such as the heart. Venous drainage from the heart has little reserve oxygen. Because of this, the myocardium is flow dependent for its oxygen requirements. Any change in the oxygen demand by this tissue bed is met primarily by a change in the flow through the coronary vessels. Other investigators have studied the effects of hypoxia, due to lowered inspired  $P_{O_2}$ , on coronary hemodynamics. The purpose of these experiments was to determine the effects of hypoxia induced by sub-clinical levels of COHb on the coronary hemodynamics and left ventricular function. The magnitude of the effects was measured in the conscious dog.

## Methods

A tracheostomy was performed on six dogs by removing the ventral one-third of three tracheal rings and suturing the skin to the exposed edges of the severed rings. A doppler ultrasonic flow transducer was affixed around the left circumflex coronary artery to measure the velocity of blood flow through this vessel. A solid-state resistance strain gage (Model 1017, Whittaker Corporation, Pasadena, California) was implanted within the apex of the left ventricle to determine left ventricular pressure and  $dP/dt$ . A polyvinyl chloride catheter was placed in the left atrium to determine left atrial pressure and to take arterial blood samples for the determination of the blood gases, pH and carboxyhemoglobin. The catheter and the lead wires from the transducers were passed subcutaneously to the dorsal area of the neck and exteriorized. The electrocardiogram was determined with pin electrodes placed subcutaneously in the sternal region. The ECG, heart rate, pressure and flow signals were monitored with an oscillographic recorder and recorded on magnetic tape. The data recorded on magnetic tape were analyzed with analog and digital computer processing.

The blood gases and pH were determined with a pH and Blood Gas Analyzing System (Model 113, Instrumentation Laboratories, Inc., Boston, Massachusetts). The concentration of COHb in the arterial blood was determined by liberating the CO bound to the iron of hemoglobin and measuring the content. This was carried out in a closed glass vessel under an atmosphere of nitrogen, figure 1. Blood was anaerobically placed in the flask and the hemoglobin oxidized with an acidic potassium ferricyanide solution. The liberated CO was purged from the reaction mixture with nitrogen and collected in a tonometer. The CO content of the collected gas was measured with a non-dispersive infrared spectrometer (Lira Model 200-S Mine Safety Appliances Company, Pittsburgh, Pennsylvania). The percentage of the hemoglobin that was completely saturated with CO (%COHb) was calculated by dividing the CO content by the CO capacity. The latter was obtained from the Hb concentration which was determined spectrophotometrically as cyanmethemoglobin.

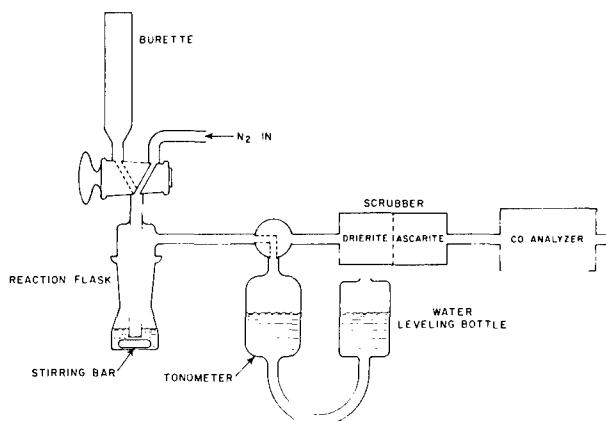


Figure 1. EXTRACTION AND ANALYSIS SYSTEM FOR MEASURING CO IN BLOOD.

After the surgical preparations had been completed, the dogs were permitted to recover for two weeks before they were exposed to CO. During this period they were trained to lie quietly on an examination table and to accept an endotracheal tube. This was accomplished so that the responses to CO could be measured in the conscious state.

At the end of the recovery period, control measurements were recorded and each dog was exposed to CO-air mixtures for approximately 30 minutes. The CO-air mixtures contained either 1500 or 3000 parts per million (ppm) CO in room air. The 1500 ppm mixture produced a linear increase in the concentration of COHb of about 1%/minute and was used primarily throughout the study. During the exposure, the cardiac function data were recorded at five minute intervals and corresponding arterial blood samples were drawn. Two separate exposure experiments, 4-7 days apart, were conducted on each dog. The time allowed between experiments was to assure that a complete washout of the previously absorbed CO had occurred.

## RESULTS

The computer processing of the cardiac function data produced a printout record as shown in figure 2. It is the result of 10 cardiac cycles taken between R-R wave intervals. Shown on the abscissa is time in milliseconds. On the ordinate, in the upper panel, is shown the left ventricular pressure curve, starting at systole. The second panel is left ventricular  $dP/dt$  and represents the rate of the pressure increase. The maximum amplitude of this derivative is generally used as an index of the force of contraction of the myocardial muscle. The third panel shows the velocity of blood flow through the left circumflex coronary artery. The velocity can be converted to volume by measuring the diameter of the vessel and making appropriate calculations. It can be seen that the major portion of the coronary flow is during diastole. This is also shown on the forth panel, which is the coronary stroke volume. It is the integral of coronary blood flow.

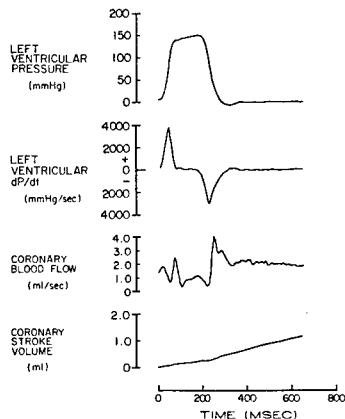


Figure 2. COMPUTER PRINTOUT OF THE CARDIAC FUNCTION DATA UNDER CONTROL CONDITIONS.

The changes observed as a result of increased levels of COHb were very striking. The observed increase in coronary flow was linear with %COHb. Five percent carboxyhemoglobin caused a 14% increase above the control value for coronary flow. Twenty percent carboxyhemoglobin caused a 57% increase in flow. This was significant to a P level of 0.001. Coronary stroke volume rose to 8% above the control value with 5% COHb and to 37% greater than the control value with 20% COHb (P = 0.01). This increase was also linear with % COHb. The heart rate increased 4% above control at the 5% COHb level and a 20% increase was observed when 20% of the hemoglobin was saturated with CO. There was no significant change in left ventricular pressure or dP/dt either at 5 or 20% COHb.

#### DISCUSSION

The results obtained in this study indicate that low levels of carboxyhemoglobin can cause significant changes in coronary blood flow. Hypoxia induced by the elevation of COHb appears to have a different effect on the cardiovascular system than hypoxia induced by lowering the inspired oxygen. If the inspired  $O_2$  is lowered, the arterial  $P_{O_2}$  is decreased. Under this condition, an increase is observed in coronary flow, heart rate, coronary stroke volume, and left ventricular dP/dt. When the inspired oxygen is held constant and the COHb is elevated, there is no change in the arterial  $P_{O_2}$ . An increase is observed in coronary flow, heart rate and coronary stroke volume but no changes in left ventricular dP/dt occur. The difference between the two modes of hypoxia is in the contractile force of the myocardium. The mechanism involved in the changes observed does not seem to be related to arterial  $P_{O_2}$ , since the carotid and aortic chemoreceptors are not known to be stimulated by increased concentrations of COHb. However, the heart rate increased with both modes of hypoxia, suggesting that the mechanism may involve central nervous system structures. The difference in the response of left ventricular dP/dt to the two modes of hypoxia may be due to the metabolism of oxygen. Carbon monoxide combines with myoglobin and with cytochrome oxidase of the electron transport system and thereby may inhibit the utilization of the available oxygen in the myocardium.

## DISCUSSION

MR. VERNOT: One question. The arterial  $pO_2$  was measured?

MR. ADAMS: Yes.

MR. VERNOT: And there was no change?

MR. ADAMS: That's right.

MR. VERNOT: I asked the question because I've seen it stated before and have seen some theoretical reason for the statement, although I am not prepared to go through the reasoning, that blood with rather high or moderate concentrations of carboxyhemoglobin is subject to lower  $pO_2$ 's in arterial blood.

MR. ADAMS: Our data do not support that.

DR. DU BOIS: Adjustment of local blood flow in this particular organ, coronary flow, seems to allow the heart to make up for the increased work that it would require. First, it has deprivation of oxygen to its own self and secondly if you have an increased cardiac output, it has to beat harder. A question on cerebral blood flow would be whether there is anything about the cerebral vessels which would lead to similar action in dilating in response to some CO. And whether this would account for the differences in the psychological tests--under one condition getting an adaptation of cerebral blood flow and under another condition not getting it at that time.

MR. ADAMS: This is an acute exposure, we don't know what happens in a chronic situation.

MAJOR MAC KENZIE: Would you care to comment on what percentage of the cardiac reserve this represents?

MR. ADAMS: This is a subject for long debate. We haven't taken these dogs to their maximum capacity, I don't think I could comment on that in the dog. The other thing is, how do you relate from the dog to the man as far as the pulmonary cardiovascular system is concerned? In the January meeting of the New York Academy of Sciences it was speculated by Ayers that there may be a greater effect on man than there is on the dog.

DR. HODGE: Were there changes in the heart rate in other animals or people with 5% carboxyhemoglobin?

CAPTAIN O'DONNELL: In the studies that we reported last year we did just simple measured pulse and there was no overall change in heart rate. The only thing that we did find was that there was a change, a very slight change, in the response of the heart to the onset of a stimulus. In other words, when the subject has to start tracking, the difference between his 10 second pretracking and the first 10 seconds of tracking was different in the first 15 minutes of exposure, but not after that. Now at that time we considered this a very tenuous result and did report it, but we had no mechanism to explain it.

MR. ADAMS: We were real surprised to see that all of our data came out linear and we really scrutinized it hard because it does look fishy--you know as if it's done by computers.

DR. BACK: We didn't do any acute work but we've done quite a bit of work on the long term exposure. Dr. Paul Chikos went into the domes and measured heart rates and blood pressures and so forth, and we didn't find any changes attributable to the CO<sub>2</sub> between preexposure and during exposure. We can't, we don't, have a group of controls running around with 70% hematocrits, that's as far as the animals were concerned, but there was no great difference in their heart rate. He tried to find out whether there was a difference between animals on the ground and at altitude. There was no difference there either.

## DISCUSSION

DR. HODGE: I've asked Dr. MacFarland from Toronto to expound a little bit further on the point that he was making this morning.

DR. MAC FARLAND: I'll begin by apologizing for taking your time, but the point that was raised from Lt. Rose's paper on which I commented very briefly this morning and probably in a rather confusing way is one of rather considerable theoretical interest and is one of practical interest too with carbon monoxide, specifically, because of this curious fact that when you have exposed animals to carbon monoxide for a fixed period of time and then terminate the exposure, your animals now begin to recover. Now this is rather different from what we usually see with toxic agents. You put the animal into an atmosphere of, let us say,  $\text{NO}_2$ , or chlorine or phosgene or what have you, and expose him for a fixed period of time and take them out of the chamber, he is not now beginning to recover, he is now beginning to die and a few hours later they all pop off, or some fraction of them pop off. So carbon monoxide is unusual in this respect, if you are going to kill them you have to kill them while they are in the chamber. This introduces a complication, however, in determining the  $\text{LCT}_{50}$  and consequently a bit of difficulty in determining the  $\text{LC}_{50}$ . I just wanted to mention that in the case of carbon monoxide the thing to measure is the  $\text{LT}_{50}$ . This is a much better parameter to try and get at, in this particular case. I want to take just a moment to explain where the difficulty enters into this thing. I have drawn a hypothetical graph here and I've tried to make it conform a little bit to the data that Lt. Rose presented. I believe you worked with around 2000 parts per million and figured this was about the four hour  $\text{LC}_{50}$ . Now this part that I've shown is a percent mortality scale against the time scale, and I am envisioning, just for the sake of simplicity, that we have groups of ten animals, and consequently if we observe a percent mortality, it must move in jumps of 10%. We imagine that at 2000 parts per million we put animals in the chamber and start exposing them and what we will observe is that we get a distribution of survival times, and curiously enough, it is one of these skewed sigmoids which looks exactly like the kind of curve you get when you do the  $\text{LC}_{50}$  determination, with other types of materials. I have done this type of experiment with carbon monoxide and what you find is that it takes a while before they begin to go and then you also have in any given group of animals a distribution of susceptibilities. So the weakest fellow goes to the wall first of all and he is the fellow you see here. He represents a 10% mortality in a group of 10 animals and we will suppose that he dies after 2.6 hours, which I am just reading off roughly from these scales, in the course of this exposure. A little while later the second fellow pops off, and I put down again a guess at the time, say 3.2 hours, and so it goes. Now if you care to carry on with this thing, you'll wish you hadn't after a while, because what happens is this: eventually the sixth

animal goes and then quite a long time after, the seventh animal goes and then a long time after, the eighth animal goes, and you begin to wonder whether that last animal who is obviously the least susceptible or the most tolerant, or the hardiest in the bunch, is ever going to die, but he does finally way out here somewhere, and this could easily be 8, 10, or 12 hours, and it's a long time to wait. Fortunately if you want to calculate  $LT_{50}$  data, you only have to carry the experiment through to the point where the sixth animal has died, then you have enough to push this thing through the mathematics and deduce the  $LT_{50}$ . Now where the problem comes in is this, let us imagine an experiment that terminates at four hours and let us suppose, at this concentration, this in fact has been the survival performance of these animals, which I think probably conforms reasonably well to probably the sort of observations that Lt. Rose made. Now the problem here is this. I won't say the dose that the animals received because of this fact that the CT product that these animals have received differs for each animal and this is perfectly apparent. Number one on the hit parade here, his CT is  $2.6 \times 2000$  parts per million-hours, and this fellow's is  $3.2 \times 2000$  and so on, and so on. So that each one of them is different, each animal has in fact received a different dose in order to kill him, and the most susceptible animal dies with the smallest dose. Now this is contrary to what you do in an  $LC_{50}$  determination, where the animals do get a fixed CT product, when you calculate the  $LCT_{50}$ . Here each one of these animals is different and you can't really average this thing, so the thing that you can derive from this data is not an  $LC_{50}$  but rather an  $LT_{50}$ , and in the instance that I have put on the board here, the  $LT_{50}$  is probably about four hours. I don't know if I've explained this thing to you or just confused you further, but it was a point I wanted to make.

DR. BACK: You only made one other point there that should be made--you said that the animal that gets  $2.6 \times 2000$  is different from one that gets a different total dose than the  $3.2 \times 2000$ . That's only true if they all breathe at the same rate and with the same depth. You're telling me that all rats, the guy down there at 2.6 is breathing the same rate as the one up there at 3.7 and that may or may not be so.

DR. MAC FARLAND: Well, I'm assuming the usual sort of alveolar conditions for this experiment. These ten rats that I used in this experiment are randomly selected from a larger population, they have a very narrow weight range, perhaps they all weigh  $225 \pm 5$  grams, and they are all normal animals. That is true and under these circumstances, you can detect small differences in respiratory rate, etc., etc. But, to a degree of approximation, these animals are uniform, and differences in the minute volume in animals of this sort do not produce significant changes in the location of these points.

DR. SALTZMAN: This point that has been raised is on the fringes of a very interesting theoretical problem, that as we must recognize, the laboratory exposures to constant concentrations are not realistic and, in actuality, for example if a person is in traffic being exposed to carbon monoxide, he is exposed to a very rapidly fluctuating concentration. As far as the air pollution problem is concerned, if you ever saw a monitoring chart with a good, rapidly responding instrument, you would see that the concentrations of many pollutants fluctuate very, very rapidly and erratically. Then

this raises the question of how we interpret or analyze the data in relationship to the laboratory studies. Now we can divide this fluctuating concentration into an average value for the whole period, then superimposed on this you might say there is a fluctuating component. This can be analyzed mathematically. It's a rather complex problem, and the gist of the final solution, which incidentally is in a paper I wrote that will appear in the Journal of the Air Pollution Control Association in the next month or two, is that the body, or for that matter a sampling device or sampling system, does not see all the fluctuation. The rapid fluctuations are damped out; in essence you could say you are looking at the world through a colored window which only transmits certain frequencies. In the case of the biological picture one of the significant parameters is the biological half-life of the contaminant in the body, and the fluctuation period ratio to this parameter is a significant one. If it's a very rapid fluctuation with relation to the biological half-life, it is not seen by the body. In the case of manual sampling procedures, the length of the sampling period is important. The main point that I am getting at is that we have to recognize that these laboratory studies at constant concentrations are certainly not realistic with regard to exposures.

LIEUTENANT ROSE: Only to reiterate that these exposures were designed for a very specific type of situation, one of a closed breathing atmosphere under conditions of elevated pressure, which, of course, would not be any type of environmental situation that one would encounter on the streets, in automobile traffic, or anything else. This was designed expressly to augment existing information for the Manned Exploration In The Sea Program. These men are exposed for long periods of time under these conditions, and if carbon monoxide is being generated, it is being generated more or less constantly and they will be exposed to a more or less constant concentration of it. Therefore, we have to get some indication whether or not the toxicity of the gas was altered under conditions such as these.

DR. BEARD: I think Dr. Saltzman is quite correct in what he says, but I think a further extension of this simply is to say in the long run, at least as far as looking for effects in humans are concerned, we must rely upon epidemiologic studies, and that our laboratory studies are simply the groundwork on which to build competent epidemiologic observations. We need solid groundwork based on these unrealistic laboratory exposures in order to be able to know what to look for and how to do the measurements in epidemiologic studies.

DR. THOMAS: This is in no way an argument, we all realize that we go to great pains in trying to conduct experiments where we can cut out unnecessary numbers of variables. We are not really in the air pollution business. What the Air Force is after is to take a steady concentration of some agent and see what is the maximum tolerance. Once you've got those data you can say, O. K. imagine that that steady concentration was the peak, and there you are at home, free. But is it meaningful to try to vary concentrations rapidly up and down in inhalation exposure studies? I wish Dr. Hodge would comment on that.

DR. HODGE: I think anybody who has ever done inhalation work shudders just at the thought.

DR. AZAR: I would like to comment on some work that was done by a graduate student at the University of Iowa in the past year, where he took human subjects and exposed them to varying concentrations of carbon monoxide. He integrated the end-tidal air concentration as he varied the concentration, he compared it with a constant exposure trying to look into this very fact of changing concentrations of low levels and when you integrate the area under the curve of excreted carbon monoxide in end-tidal air, he did not find any difference in the pattern of exposure at these low levels. Now he saw some suggestion that whenever the difference was quite marked there may be an effect, but he was trying to keep the concentrations in levels that might appear in traffic.

COMMANDER BARBOO (Navy Department): I was most interested in Dr. Schulte's attempts to assuage the disparity in the various experiments and was disappointed that it was not addressed less to the carbonyls and more to the reflex response of the psychologist. If we are using adults and these largely react in a reflexive manner in this world and these follow certain neuronal pathways this leads me to ask Dr. Beard with your sixteen symbols you equated them with numbers, I understand, in the learning process?

DR. BEARD: The reason for choosing the abstract geometric symbols was to break away from the established habit patterns of an alphabet or a series of numbers. Now, actually the four subjects in each instance very quickly developed a strategy for analyzing what they saw on the screen, and in each instance this was the development of some kind of an alphabet, if you will, in which they simply equated the symbols with numbers or letters, and they had various different games that they played. One of them was rather clever. She made up little sentences in which she characterized the symbols as there's one which is an inverted T and then there is a square, and she would make up a sentence saying "We had tea in the square", and then went on to the triangle. That was the way she organized her thinking.

COMMANDER BARBOO: I'll allow my ignorance to spill out all over the floor. Were you to set the pattern, let's say the next day or in the next hour that they must relearn the pattern. They must unlearn the former conditioned response. You did also do this, I understand? And this would in some way attenuate this reflex, that they must then relearn. When you relearn, you go through a different neuronal pathway, it's not reflexive--you go up and down and back again and double check, that you're not getting the former pattern set, or am I being unclear?

DR. BEARD: No, you are quite correct. This is precisely why the experiment was set up in this way, so as to break away from the established reflexive patterns.

DR. DOST: I would be inclined to offer sympathy to anyone who has to work with carbon monoxide because with all this careful work and no really significant finding in terms of differences at least, certainly the lack of differences is significant, this must be pretty frustrating. To take the classic case of the traffic officer who comes to work in the morning, pretty mild mannered and stands there in the middle of the street all day and by three o'clock in the afternoon, he is becoming more and more irritable and not just from the stress of his job but from the continuous inhalation of carbon monoxide. He is becoming pretty nasty to the traffic that is swirling around him and in turn, of course, they are becoming more and more nasty themselves. Then he goes home and pounds on his children or his wife or whoever is nearest. It suggests possibly that the objective and calculated observations that are being made might necessarily have to be supplemented by some highly subjective observations of individuals of this sort in an effort to localize some sort of patterns of behavior. This sort of approach isn't necessarily as rigorous as the measurements that are being made but it would seem to me that it might bear more fruit in view of the bucket of worms that appears to be emerging with more systematic work. Any of you who have been fighting this problem have any ideas?

DR. STEWART: I don't think the bucket of worms is quite as ominous as you indicate. In our particular series of studies on an hourly basis, we ran through a questionnaire of subjective responses, so that we had a pretty good feeling when people were exposed to 100, 200 parts per million as to whether or not this was having an impact upon how they felt about life in general or anything else. So I think we have some good feeling here that carbon monoxide below 100 parts per million probably is not making somebody crabby, changing or altering personality. I don't think we have any information at all that would indicate that this would be the case. So, in this instance, I think we are in pretty good shape. What's emerging and what may appear superficially to be a bucket of worms, are some of the differences between the experiments. But I think one can lay back and look at them, rather objectively now, and, in part, explain some of the differences. For example there are three things right now that I think would straighten out the differences in observed experimental results between Rodney Beard's series of experiments and our own, and first of all, there is the double blind versus the single blind aspect. I just don't think you can go with a good single blind experiment. I think these sorts of things have to be double blinded, or you are going to end up with data that you really don't know, really can't trust. I think the second thing that troubles me is that if you are actually going to expose someone to carbon monoxide you have to know what is the amount of carbon monoxide that the individual absorbs. And I think one has to monitor accurately the carbon monoxide within the individual and the only reliable way that I know of doing this really is to measure the amount circulating in the blood stream. To do it via an alveolar breath sample introduces a fair margin of error. And then the final thing that bothers me is one has to know, has to define, the total environment in which the experiments go on. He has to know what the other contaminants are, including control of things such as temperature and humidity.

DR. SCHEEL: I would like to ask a question of the panel in general and I would like to have a least a comment from Mr. Vernot, Dr. MacKenzie, and Captain O'Donnell. We've seen demonstrated this morning the difference between acute exposure and rate of change in carbon monoxide content inhaled and in the body and conditioned carbon monoxide inhaled and in the body. Based upon your observations of going in the direction from nothing to something, what would be your assessment of what would happen if you went from something to nothing in terms of carbon monoxide content in the tissues? Is this a stressful phenomenon going from something to nothing or not?

MAJOR MAC KENZIE: Based on clinical observations of dogs that were removed from the domes and held for hematologic studies after the exposure, there was no stress whatsoever. Their hematocrits slowly returned to normal--that was the only change.

CAPTAIN O'DONNELL: Behaviorally I would have no basis to predict what would happen. Based on what we saw going from nothing to something, I would say that I would expect no changes.

DR. SCHEEL: What about the hemoglobin that gradually went back to normal? After all, we had a viscosity increase and so forth, and this took two weeks in the dogs and the monkeys to become real. Now in man, we get the same kind of response in a heavy smoker, if we take him off his cigarettes, is his response to this partly the stress of withdrawal, going back to normal hemoglobin?

MR. VERNOT: You mean withdrawal symptoms normally associated with quitting cigarette smoking? I haven't the slightest idea.

DR. BACK: This is what I started to say before Dr. Dost started talking about the traffic cop. We can't talk about mixed effects. When you do that, you're lost. The traffic cop is being exposed to carbon monoxide, aldehydes, ketones, gasoline, kerosene, and Lord knows what else--SO<sub>2</sub> and NO<sub>2</sub> and everything. Cigarette smokers are being exposed to carbon monoxide, NO<sub>2</sub> and nicotine and who knows what else, so I don't think we dare talk about mixed effects. We can only talk about carbon monoxide. The effect of carboxyhemoglobin is a very rapidly disappearing one. The CO hemoglobin just goes and it goes in a matter of hours. If I'm not mistaken, Ed, didn't we find in some of our 400 part per million exposed animals that, by the end of a two-hour nonexposure, they were down around 3% saturation?

MR. VERNOT: This was with 100% oxygen. It goes faster when you've got 100% oxygen, normally.

DR. BACK: Right, but at any rate even in non 100% oxygen you're almost down to zero carboxyhemoglobin levels certainly within 24 hours. And I think this is what the Navy found in their exposures. And this Lt. Rose didn't bring out in this paper but I happened to review his paper, but if he had killed these animals, instead of decompressing them, then he would have gotten carboxyhemoglobin levels probably all the same, or very close to being the same. And this he didn't do, unfortunately, he chose to bring them back alive, if you will, rather than counting them and then re-

compressing them, and killing them right then and there and grabbing the blood in a big hurry. The point of the matter is that CO hemoglobin is gone within 24 hours. Now the hematocrit and hemoglobin totals are still relatively high, but it doesn't take long for that to wear off.

DR. DOST: Perhaps I didn't state my point clearly. I don't necessarily advocate stationing someone to observe a traffic policeman in action, but I think the possibility of maintaining people at work under controlled atmospheres and under observation of a subjective nature might assist in locating behavioral problems that arise with CO.

DR. INGRAM: I wanted to make a comment about the blood changes that were involved. What we saw this morning in the animals that were exposed over a fairly long period of time, was a very definite increase in the red cell, hemoglobin, hematocrit, and so forth which stabilized after a while. But this was a significant increase and it raises some very interesting questions because in looking at these blood picture, it was observed that there was no change in the mean cell volume. Now this immediately raises the question about what the mechanism of the increase is, and if I may be excused, I will go through some very elementary review of how red cells are made. We have a stem cell here and it gets a signal, which is the hormone erythropoietin, which is produced by the kidney in response to tissue hypoxia. This hormone, as we now know, acts primarily by inducing enzymes required for hemoglobin synthesis in this cell. And it now knows it is going to be a red cell and not a white cell or platelet. Now it begins to synthesize hemoglobin and the cell goes through a well-characterized series of divisions, making hemoglobin, specializing as they do till it gets over here. Now the thing that turns off the divisions is that the cell reaches a critical concentration of hemoglobin and beyond that there is no further cell division. It just matures and then goes into the blood stream. Now this is a dose dependent phenomenon, if you give a lot of erythropoietin, you synthesize hemoglobin very much faster than if you give a small amount. So far this seems to be the primary control for red cell production. Now it would be quite reasonable that you would produce erythropoietin in the animals exposed to carbon monoxide. But if so there is a piece missing, because each division results in two cells that are a little smaller than the cell they came from. I haven't drawn it very well, but that's the way it goes. Now in the normal human and in the normal dog, and probably in most mammals, if one measures cell volume on a cell by cell basis, not the mean, but one cell at a time, and plots relative frequency against cell volume, and this is a linear scale, you have a very characteristic and highly reproducible histogram that looks about like this. These are probably two normal curves, they might be two log normal curves, but so far it looks like they're two normals, but this is not materially influencing them. This volume is roughly half this volume. Now it looks then, on the basis of a lot of other studies on how these cells divide, most cells go through five cell divisions, and some of the cells go through only four cell divisions in the normal animal. These are probably the five division cells and the four division cells. Now if he is exposed to something that makes him produce erythropoietin in increased amounts, more of the cells will go through four divisions--they will start skipping because they make their hemoglobin earlier, and this curve in that case would begin to lift off here. Now, you won't see this if you look at the whole blood and if you look at the mean values, but if you use a little trick, which nature fortunately provided for

hematologists, it turns out that as the cells get older they get denser, at least they act as though they're denser. They spin to the bottom of the tube, if you spin them down at fairly high speeds in a long narrow tube. If you label a cohort of cells, when they first appear in the blood, if you look at the very top layer of red cells, all the label is there. As time goes by, over the life span of the cell they are found further and further down in the tube. So it is possible by taking the top part here to look at the newly formed cells. Now they go up here, whether they are larger or smaller than normal or normal, and in the normal animal if you took the segments of the tube, all of the volume histograms fall right smack on this line, in other words they don't go down here because they get smaller and concentrated. If you take an animal that has been stimulated and now instead of looking at the whole red cell mass, look only at the newly produced red cells, then after even a very modest stimulus, you can begin to see this curve lift off and if the stimulus is very severe, you may actually shift practically all the cells to this distribution, and if it is extremely severe and if the animal has some other problems, you may actually get some only going through three divisions, clear out here. Now this is likely to occur whenever you skip division, and I think we heard this morning that the cells are normochromic, normocytic, and I think we've agreed by doing some very simple arithmetic, we could estimate that the dogs were making cells roughly twice as fast as normal, and with 100 day life span this isn't a great increase in this fraction of cells. So you would certainly have to look here to see them at all, probably wouldn't see them in the whole column. This is very simple to do and I would like to suggest that this would be worth looking at, but I would also like to get some comments from those of you who are thinking about mechanisms as to whether there is some mechanism postulated other than erythropoietin that accounts for this quite remarkable response. If indeed these are not altered cells, we have a very significant change from all other kinds of hematological response, and it's extremely interesting.

MR. VERNOT: Dr. Ingram and I talked a little bit before lunch concerning this and one thing that I forgot to mention during my talk, which I think deserves mention, is that there was a slight but measurable increase in reticulocytes. After about four weeks exposure it became obvious, it was about a doubling which is pretty hard to see, but it appeared to be reproducible during the time of equilibration to carbon monoxide intoxication.

DR. INGRAM: One other point that is quite interesting in light of your comments about the dogs' behavior, is that we've studied this in dogs extensively, in dogs from whom we've drawn varying amounts of blood to stimulate erythropoietin production. You can predict very precisely when these cells appear. You do all the fractions and they are definitely normal and then all of a sudden this cohort of abnormal cells comes out in the blood, and it is between the third and fourth day. And it was on the third day that your dogs suddenly perked up and became lively again, which I think is quite interesting.

DR. JACOBSON: I would like to make a general comment and then a specific question. The general comment is that it seems to me that a number of the remarks made about carbon monoxide poisoning are an inability to attribute some of the effects seen to the degree of hypoxia that would be accounted for by a direct effect of reduced oxygenation of the blood. If, however, carbon monoxide has a more specific effect on the heart, and cardiac dysfunction follows, for example by lowering of pulse pressure, then the degree of tissue hypoxia seen would be greater than would be accounted for by the amount of carboxyhemoglobin, and I believe there are those and the paper of Lewy and Drabkin referred to earlier today is one that does kind of suggest, that maybe the trouble is largely of the heart. I would like to ask Mr. Vernot a specific question. I either missed or misunderstood your description of the chamber atmosphere and I was wondering if you would repeat that and state why you selected that combination of oxygen and nitrogen.

MR. VERNOT: Well, we worked under 260 millimeters of mercury pressure, 68% oxygen. And this is a space cabin atmosphere essentially, and we were working under those conditions at the time. We were interested in the effect of carbon monoxide under space cabin conditions. Dr. Thomas has a word to say.

DR. THOMAS: This work, ladies and gentlemen, was started because we have a manned space program in this country and the question came up "Just when do you have to abort the mission?" And I think it is darned important that we have a body of knowledge there where we can say you don't have to abort, even if you have to pay the compensatory mechanism. As the austerity increases, aborting a mission becomes pretty darned expensive. Now, what we have seen, and there is my pathologist sitting there, is that there are no arteriosclerotic plaques; you have the subjective feeling that the dogs' heart or the monkeys' heart is a little thicker in the left ventricle, but when you measure it there isn't anything. But these studies are for a manned space mission, they aren't for the Public Health Service; they don't consider the sickly, elderly, the asthmatic, the coronary patient. Please keep in mind, that we are working for the Air Force. We are working for certain space missions.

MAJOR MAC KENZIE: I would also like to make a short comment on the work of Lewy and Drabkin and also Lewy and Eric. They found heart lesions in their control dogs too, so this very well could have contributed to the CNS lesions. Also, they were using street dogs, at a time when we didn't have the knowledge in veterinary pathology that we do now. Also, I suspect from the descriptions that they gave of the lesions that around the eighth week something went wrong with their exposure chamber and the dogs were gassed rather heavily, but they did have compromised hearts. I hope that I made the point clear this morning that we were using healthy robust animals and we weren't talking about the compromised heart or cardiac cripple. Even with these healthy animals, my feelings are that the limitations on the tolerance to carbon monoxide rest more with the circulatory system, considering there is a functional hemopoietic system, they rest more with the circulatory system than anything else.

DR. SCHEEL: I wanted to ask Ken or some of the people here whether they had any tissue respiration studies done on sacrificed animals after this accommodation to a high carbon monoxide content and especially heart tissue, to find out whether there is actually, in fact, anything to indicate an inhibition of the cytochrome system?

DR. BACK: The answer is that on brain tissue we've done work at 200 parts per million and 400 parts per million in monkeys on pyridine nucleotides and  $pO_2$  ratios, and there were no differences. These were animals exposed for 14 days, as I remember. I wish I had the data, but some of them were exposed for 7 days and some of them for 14 days, and as I remember the data, there were absolutely no differences in  $pO_2$  ratios. DPN and DPNH remained unchanged, TPN, TPNH remained unchanged, as I remember the data.

MR. WANDS: I am going to ask the Wright-Pat people, particularly, if during their chronic exposures, any of these dogs or the monkeys, or any of the other animals were given a work stress. Were they put on an exercise cycle, like Dr. Beard did with some of his human patients, or were there any other physiological or physical stresses of that sort? Do they still have a good reserve work capacity?

MAJOR MAC KENZIE: Sergeant Hunt who was NCOIC of the laboratory did most of the blood drawing and at the end of the experiment in the CO dome, he stated that he wasn't sure who was going to end up on the table, the baboon or him.

DR. MAC EWEN: We didn't make any attempt to deliberately work the animals. In the domes, the dogs are divided into two cages in groups of four, and they exercise themselves, they run, they romp, and they frolic and they fight and they show their libido, and as somebody mentioned earlier, a baboon in one cage in one dome let out all the monkeys and they put on quite an exhibition for us. We had quite a time getting them back in, in fact. We have the same sort of activity in the monkeys, the same kind of troubles handling the monkeys in the high level carbon monoxide as we do in ambient conditions, and that's about all we can tell you, we have made no attempt to measure activity in mice. Dr. Thomas looked at the activity in dogs and I'm not sure if that's going to be covered in his paper this afternoon or not.

DR. THOMAS: That carbon monoxide study was a rather lengthy one, if you know what it means to take time-lapse photography every single day, I didn't get through with it yet, I think next year we'll have a pretty objective measure of continuous activity. As Major MacKenzie explained to you, these animals were clinically symptom-free. As a matter of fact they were more vicious probably than the controls and I'll be very much surprised if I see a depression of continuous activity but I don't have the data.

DR. MAC EWEN: I would like to make one more comment on the purpose and some of the significance of the work that was done here at Wright-Patterson on these long term high level exposures at reduced pressure. As Dr. Thomas said this was done with concern of long term space missions. Now on a long term space mission, such as a flight around Mars and back; if you get out there, three months out, you just don't abort, you have to come back. You turn around and it takes at least three months or perhaps a little more because home isn't where you left it. So, if a carbon monoxide concentration begins to build up, beyond what we normally consider, or have established as abort levels, what do you do? You don't jump out, you have to fly home. And what we've tried to do here and I think have shown, is we can tolerate rather high concentrations. There is another alternative, we can vent the atmosphere of the cabin to space and replace the atmosphere, but the astronaut has a limited amount of that aboard his vessel and he can't keep doing that every half hour or so. He must retain his oxygen and clean it up, as he does to get rid of the carbon dioxide and some of the objectionable odors, and he cannot just keep dumping to get out CO. I think that we have shown that you can tolerate rather high CO levels, a good healthy man, if they are built up slowly, and you can acclimatize or adapt to this, whatever you want to call it by building up your red blood cell count and the corresponding amount of hemoglobin. You can get adequate tissue oxygenation to survive, and that's really the important thing, survival, in this experiment.

DR. BEARD: May I shift back to discussion of some behavioral things because I've been very eager to hear a little more from Dr. Stewart and Captain O'Donnell. First comment, I would add a couple of things to the explanation for differences of observations, among the several experimenters. One is that I must say I have always been emotionally unable to believe what objectively we reported concerning the small amount of variation among our subjects in our first experiments. The correlations are just too good to be believable really, we've checked this over and over and can't find any flaw. I will be surprised if we ever can again get as excellent correlation or that anyone else ever will. There is a factor of individual variation, which I think it is rather extreme and sometimes it's variable. We have a subject who was used in early experiments on this display test, who distinguished herself by the excellence of her performance. She got very high scores and became very stable. That was on somewhat less difficult tests. When she was put on to the fifteen item sequence, she fell apart completely and this was consistent over a period of time. This led to some rather detailed questions and we found that in the interval, she had begun to use marijuana, not frequently and not very much, but there is one of the psychologists in Berkeley who suggested at least that pot can have prolonged effects and I don't offer this as evidence to support that view, but simply as an interesting observation and an observation that a subject who appears to be performing very well may change. Second, I would bring out the possible differences in groups. We have one medical student subject who distinguishes himself by having a very high performance level and even when he is in a test situation where he gets no feedback as to his results, he is constantly trying to better his score, using only his own subjective impression of the kind of score which he has made, and I think that it is going to be very hard to show the effect of carbon monoxide in that subject. I wonder if this may not account for a proportion of the results that Dr. Stewart reports. Dr. Stewart says that he emphasizes that we must

know the amount that the individual absorbs. On this I had a little question, it seems to me that his own observations indicate that in these healthy young subjects that there isn't very much variation in carbon monoxide hemoglobin level given the same conditions of exposure and the same amount of activity in the subjects, so that I wonder if great precision in measuring the blood CO hemoglobin level is quite so important. Finally, I would only make a point that there is a very tricky business of trying to measure an effect where one is looking at a customary activity--we've talked a little bit about this before. The group at Cambridge in England has studied this rather extensively and it's their suggestion that one way to get at this is to have the subject carrying on his usual activities and then to add an additional task and the additional task is the one which may be subject to some variation under stress. Now in the test that Captain O'Donnell used, I think that this was taken into account and I think very nicely done. But, Dick, what about your feeling as to population variations?

DR. STEWART: I think we'll probably have to look at different segments of the population and see whether or not the medical student or the graduate student or medical school faculty happen to be unique in their desire to perform well. I don't know if you have taken your own test or not, the only danger with that test that I can see is someone with low level motivation tends to fall asleep. Actually, I think when the series is finally extended long enough that it may well show that people perform better in the audiometric booth than they do out in a group situation where there are additional, always additional little distractions. As to the question about precision in measuring carboxyhemoglobin, I think it is absolutely critical that it be done. Just because our particular little group had the same degree of sedentary activity, it happened to be a very homogeneous group, doesn't mean that somebody else's group or a student will do the same especially if they are tested, one by one, where they might be under different emotional stresses at different points in time, and breathing differently, and so on. I think it is absolutely critical that the carboxyhemoglobin be accurately monitored, or we will never be able to compare one experimenter's results with another. And I would think that right now, Rodney, you have a golden chance since you have begun to use a visual stimulus instead of an auditory one with the same test, to go back where you have the no-effect level with light to see whether or not you get an effect with sound. I think that is critical at this point because it will enlarge your population, and as I understand it, you are now monitoring breath.

DR. BEARD: Not very successfully.

CAPTAIN O'DONNELL: I think also with respect to this carboxyhemoglobin determination, we were very surprised to find such low levels during sleep. Now we had carefully calculated CO exposure levels so as to achieve certain carboxyhemoglobin levels but we never got to them. So in that case it was very fortunate that we did have an accurate measure.

SESSION II

METHODOLOGY

Chairman

Mr. Ralph C. Wands  
Advisory Center on Toxicology  
National Academy of Sciences  
Washington, D.C.

## A SYSTEM FOR DILUTION OF ROCKET OXIDIZERS

Stanley D. Erk, Sr.  
and  
Lloyd W. Kaczmarek

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

### INTRODUCTION

The sparsity of toxicological data covering various rocket oxidizers such as oxygen difluoride ( $OF_2$ ) and chlorine pentafluoride ( $ClF_5$ ) is apparent even in the most thorough reviews of the literature. It is likely, therefore, that more studies will be initiated in toxicology laboratories as interest and use of these chemicals increase.

Inasmuch as the rocket oxidizers are highly reactive and extremely toxic (Sekits, 1963; Weinberg, 1967; Levine, 1965), it is much safer and easier to handle dilute concentrations rather than the pure material. Therefore, a facility was designed and constructed to perform vapor phase dilution and storage of oxidizers with minimal toxicological and reaction hazard to operating personnel.

### SAFETY EQUIPMENT

The dilution system was assembled in a building modified specifically for this purpose in a relatively isolated location. The building was constructed of 18" to 24" thick concrete walls with steel reinforcing rods and a wooden "blow-off" roof. All enclosed electrical fixtures were rated as explosion-proof. Also located within was a wet type fire sprinkler system, an emergency eye wash fountain, emergency shower, and control switches for a warning system to inform outside personnel of potentially hazardous conditions in the immediate area of the facility. An oxygen inhalator-resuscitator was placed for easy access. All actual work on pure oxidizers was done in a permanent walk-in hood (figure 1) constructed of double thickness cement block with steel reinforcing bars. The various control panels were positioned outside of the hood. A large exhaust canopy covered this area and was connected to redundant exhaust blowers situated outside the building. A steel fire door provided entry into the hood area while observation was made possible by a narrow window of laminated safety glass along one wall.

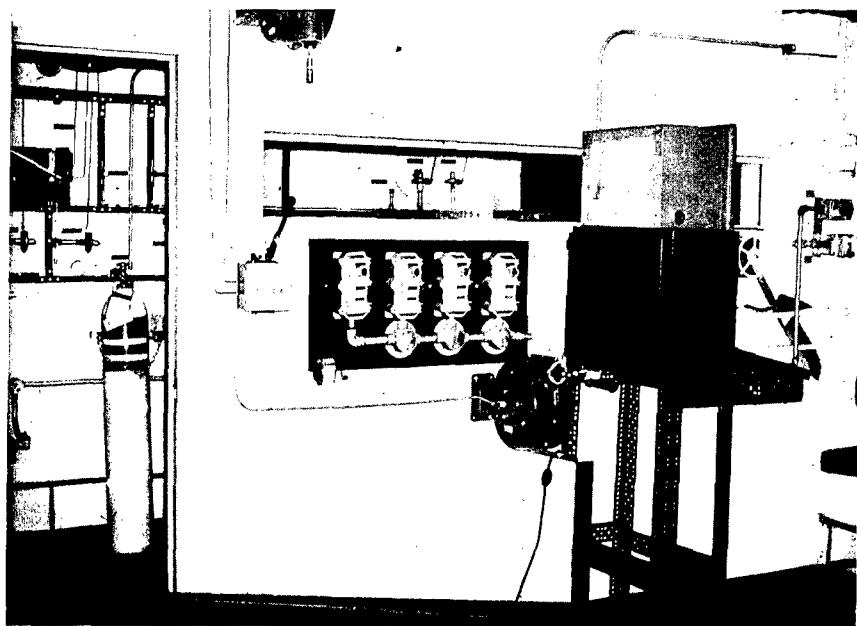


Figure 1. WALK-IN HOOD WITH EXTERNAL CONTROL PANEL

Personnel safety equipment (figure 2) included neoprene aprons, gloves, boots, and suits to be worn as needed. A cascade type compressed air manifold was installed to provide breathing air for full face mask type respirators. Voice actuated transceivers with protector headsets integrated into the full face masks insured accurate and instant communications during critical periods of the dilution process.



Figure 2. SAFETY EQUIPMENT

Safety methods and equipment were applied only to the extent necessary for a given situation. Therefore, mobility of personnel was, as far as possible, unencumbered and limited operating space was not further restricted. A false sense of security on the part of operating personnel was thereby avoided.

## MATERIALS AND METHODS

The construction materials of the dilution system were of a type considered to be compatible with most oxidizer applications.

Figure 3 is a schematic of the dilution system. All transfer lines were made from 1/4" O.D. 304 stainless steel. Flareless tube fittings, the filter and cryogenic trap were manufactured from 316 stainless steel as were the wetted parts of the Bourdon tube pressure gauges. Brass was used only in the HF trap and on the valves of the cylinders A, B, C and D.

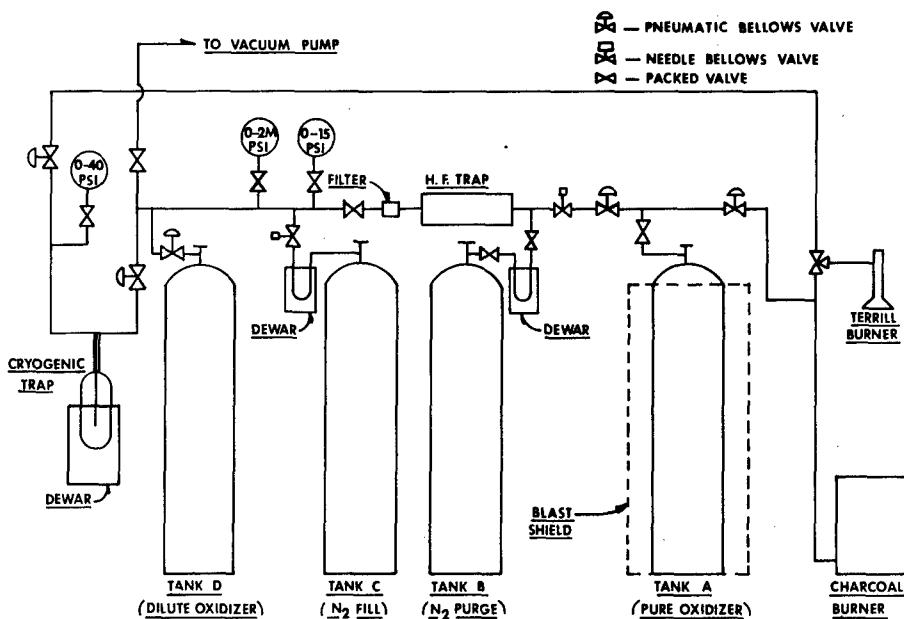


Figure 3. OXIDIZER DILUTION FACILITY

Valves used in this facility were of three types. Two of these types were welded 316 stainless steel bellows valves; one group operated pneumatically from a remote location and the other type by hand operated needle valves. The remaining type utilized 316 stainless steel in the body and stem and TFE teflon as a packing gland. While it is recognized that teflon is not recommended for moderate to severe dynamic conditions (Grigger, 1964), this system would be classified as being only for light dynamic service. All parts were cleaned, degreased, and vacuum dried in an oven at 110 C for 16 hours before assembly.

Two prepurified (99.9997%) nitrogen cylinders, B and C, were included in the system. Tank C had an approximate pressure of 2500 psi before use and acted as the oxidizer diluent supply. Nitrogen gas for purging and leak testing was supplied by Tank B, thereby leaving Tank C full for diluent purposes. Drying tubes immersed in liquid nitrogen baths ensured the high purity of nitrogen gas used.

Tank D ultimately was the storage cylinder for the diluted oxidizer. It, too, was cleaned and purged before use. Tank A was the commercially prepared oxidizer. Samples from it were analyzed for impurities by infrared spectrometry before use. The pure oxidizer was placed in a blast shield constructed from 3/8" boiler plate (figure 4). A remote control mechanical linkage opened the cylinder from outside of the hood.

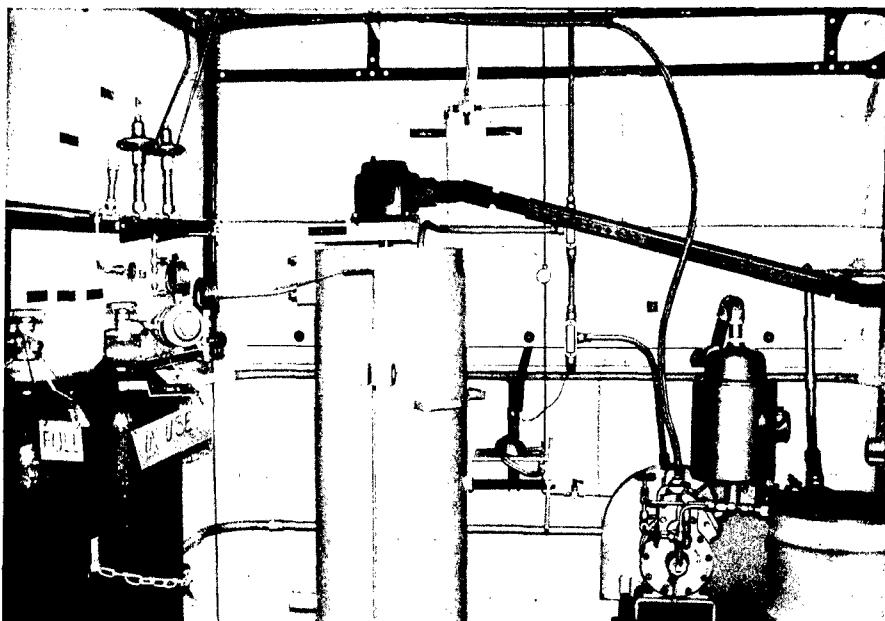


Figure 4. DILUTION SET-UP SHOWING BLAST SHIELD.

Transfer of gas within the system was accomplished by two methods. Inert purge gas was evacuated by a standard mechanical oil pump (also figure 4). Special oil for corrosive service was supplied by the pump manufacturer. Oxidizer gas was transferred by means of a cryogenic trap (figure 5). A Dewar flask, filled with liquid nitrogen, was raised around the trap causing condensation of the oxidizer within. Upon completion of the condensation phase, the trap was isolated from the rest of the system. Evaporation and venting of the oxidizer to the disposal units was precipitated by removal of the coolant from the trap.

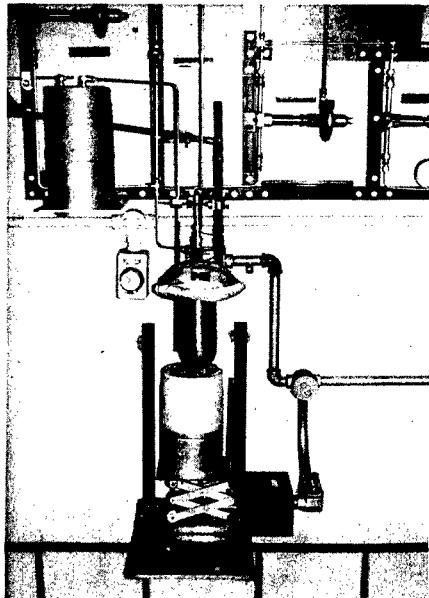


Figure 5. CRYOGENIC TRAP SYSTEM

Liquid nitrogen was remotely delivered to the Dewar flask which was raised or lowered as needed from the control panel. A heating mantel was also placed at the top of the trap. Heat was automatically regulated. Thus, evaporation of the oxidizer was aided and a stable temperature was maintained so that no leaks would develop on the fittings at the top of the trap due to uneven cooling.

Either of two devices was provided to dispose of excess oxidizer vented after passivation, from residuals after filling Tank D, or in case of emergency dumping of pure or dilute oxidizer. The first method, which was easier to use, was controlled mixing and combustion of the oxidizer with natural gas in a standard Terrill burner fitted with an additional burning stack. The second method involved passing the oxidizer through a hot charcoal burner. Most of the compound reacted with the hot charcoal, while the resultant high temperature decomposed the remaining oxidizer (Schmidt, 1967).

The dilution system was operated by combining a purge of all lines and Tank D with vacuum and pressure checks of all fittings and valves. After the system was evacuated, it was purged again and passivated with the oxidizer gas to be diluted. Upon completion of passivation, the cryogenic trap was activated which removed the oxidizer from the system and vented it to one of the disposal units previously mentioned. Proper amounts of pure oxidizer and diluent were then combined in Tank D. A final purge of all lines with nitrogen was made before the system was sealed and the completed tank removed for subsequent analysis by infrared spectrometry and delivery to the inhalation toxicology facility.

The above process incorporated a number of remote control or automatic devices. Therefore, operating personnel were not in the immediate area during the more hazardous periods when undiluted oxidizer was in the system. Temperatures during the entire process are automatically recorded at several strategic points to give advance warning of problems of a reactive nature.

#### MODIFICATIONS

Two minor incidents occurred during early dilutions of oxygen difluoride which necessitated changes in the original system.

The original system included, on the pure oxidizer Tank A, a deluxe corrosion resistant regulator constructed of monel and stainless steel with a Kel-F gasket and seat. During the initial opening of the tank of  $OF_2$  in the system, a burnout occurred in the regulator, releasing pure  $OF_2$  into the hood area. After shutdown procedures were completed, investigation showed that approximately one-half of the Kel-F gasket had burned away. This incident was concluded to be due to one or a combination of three possible causes: 1) contamination of Kel-F gasket; 2) contaminated  $OF_2$  tank valve; or 3) inappropriate regulator design or materials. In view of the uncertainty of the cause of the failure and the unknown probability of a reoccurrence, the regulator was deleted from the system.

A second incident occurred after several successful dilutions. The cryogenic trap which had contained pure oxidizer was purged with nitrogen. A small amount of oxidizer was left in the trap as expected prior to evacuation by the mechanical oil pump. It was felt that the remaining  $OF_2$  would be diluted enough so that the pump oil would not react. However, this was not the case since in rapid succession several minor explosions and one moderate explosion occurred within the vacuum pump. A relatively simple device was then installed to remedy the problem. A dip tube was extended about half-way down the cryogenic trap through a hollow fitting. The device forced the flow of gas down through the center of the trap, then up the sides and out into the system. The dip tube thus ensured a full purge of the cryogenic trap.

Since the modifications to the dilution system have been made, no problems have arisen during subsequent dilutions.

## SUMMARY

A system was designed and built to dilute highly reactive and extremely toxic rocket oxidizers safely. This system accomplished the operations of accurate pressure measurement, vapor transfer, precise addition of inert gas, secure storage of pure and diluted oxidizers, and decontamination of excess material with minimal toxicological and explosion hazard to personnel.

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## DISCUSSION

DR. DOST (Oregon State University): I have one comment on the method for scavenging waste  $OF_2$  with charcoal. We found that charcoal is an effective absorbant as well as a reactant for  $OF_2$  and as you increase temperature in the system either by increasing the rate at which  $OF_2$  is put in, thereby increasing the heat of reaction, or the heat of absorption whichever is most effective, or by increasing the heat externally, the rate of absorption increases substantially. The other thing that we found with  $OF_2$  and to a lesser extent with  $NF_3O$  was that the gas will desorb, so you have kind of a sleeper here. If you aren't careful with the charcoal then you're going to be losing  $OF_2$  into the atmosphere after you have supposedly put it away. Have you noticed this? In other words you can blow  $OF_2$  back off the charcoal.

MR. ERK (SysteMed Corporation): We really haven't noticed this as we keep our exhaust blower going all the time in our hood area.

DR. DOST: Of course, this was done on a very small scale, essentially in the IR in our case. But nonetheless it will hold, the absorption is not strong, in other words. The other thing I wanted to comment on, I think your regulator failure perhaps is due to just simply the extremely high concentration of  $OF_2$ . We had the same thing happen with  $NF_3O$ , for example, which is even more stable than  $OF_2$ , and it completely ruined regulators just by virtue of high pressure of pure material. Did you use Kel-F oil by chance in any of your procedures as a pump lubricant?

MR. ERK: No, this was a tricresylphosphate oil provided by the pump manufacturer who recommended it for this usage.

DR. DOST: 3-M manufactures an oil, I don't know how light it gets, but we have used it on certain types of pumps through which these agents potentially will go at relatively low concentrations and we know that at moderate concentrations it doesn't react, at least it doesn't react violently, and I would expect if it is acceptable for use in a vacuum pump it might be of value to you.

MR. WANDS: I would like to ask one question, Stan, before you leave. In your cryogenic trap what means do you have of avoiding an overfill of that trap with a liquid?

MR. ERK: Well, we know how much pure oxidizer we will have in the system and it is less than the trap would hold as a liquid. So we don't have that problem. You can't tell it on the slide, but the Dewar flask only comes up part way on the cryogenic trap so therefore you would only get so much liquid I would think, and so you would still have some left in this system as a gas unless you used up all your liquid nitrogen. We haven't run into this problem because we have more volume left in the cryogenic trap and we have gas that will condense into it.

FROM THE FLOOR: I saw this happen one time in a trap which was overfilled with liquid and then as it warmed up to room temperature for transfer elsewhere it exceeded the critical temperature of the liquid and this was not a very good sight to see and with a stainless steel trap like that it would be a real mess.

EXPANSION OF THE FRAGILIGRAPH SCALE BY COMPUTERIZATION  
AND ITS USE IN TOXICOLOGICAL INVESTIGATION

Henry A. Leon, Ph.D.

Ames Research Center  
Moffett Field, California

Maureen McTigue

Northrop Corporation  
Anaheim, California

George Ishiguro

System Development Corporation  
Santa Clara, California

Don Card

Ames Research Center  
Moffett Field, California  
and  
Marjorie Fuji

Ames Research Center  
Moffett Field, California

INTRODUCTION

Changes in the osmotic fragility characteristics of red blood cells have been used to diagnose and differentiate various hemolytic disorders for a number of years. More recently, osmotic fragility has been used to evaluate the toxic or hemolytic actions of drugs and chemicals as well as exotic space-cabin atmospheres. For example, the finding that pure oxygen environments can cause hemolysis was suggested by early reported changes in osmotic fragility. The use of this technique was stimulated by the invention of the fragiligraph by Dr. David Danon (1963) who has reported at these conferences in the past. As presently conceived, the fragiligraph rapidly records, in a continuous fashion, the spectrum of osmotic fragilities of red blood cells in a small quantity of blood (Kalmedic Instruments, Inc.). However, in reviewing the design of

the Danon fragiligraph it became clear that the non-linear manner of the recorded changes was less than suitable for toxicity studies and was not compatible with the accepted modes of data presentation. Nevertheless, it occurred to us that although distorted, the pertinent information was indeed being detected and recorded by the fragiligraph.

In this paper we will report on computer techniques which we will be applying to standard fragiligraph recordings for the linearization and first level analysis of data obtained from toxicologic studies.

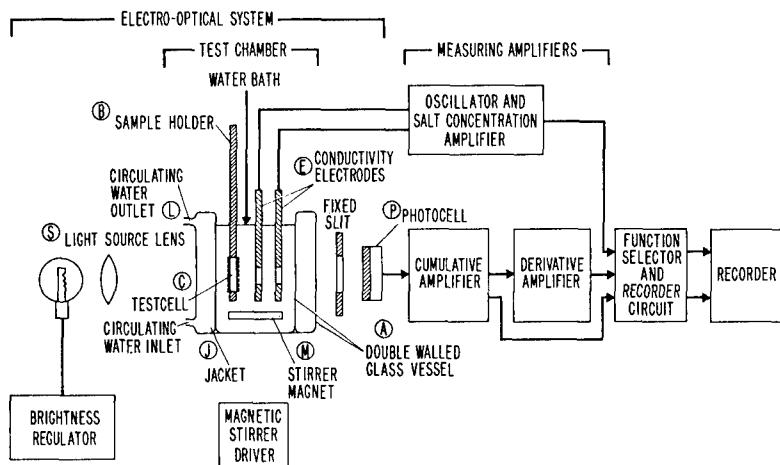


Figure 1. FRAGILIGRAPH OPERATION

### THE FRAGILIGRAPH

The basis of operation of the fragiligraph is illustrated in figure 1. A diluted blood sample to be tested is placed in a cuvette which actually has walls made of dialysing membrane. This cuvette is placed in the light path of what is essentially a recording colorimeter, thereby recording the light transmittance through the blood sample. At the same time, the cuvette is surrounded by a large quantity of distilled water which is normally thermo-regulated. Dialysis of salt-out through the membrane coupled with osmosis into the cuvette results in a continuous decrease in the tonicity of the solution surrounding the red blood cells. As the erythrocytes progressively hemolyze, reflectance decreases and transparency increases. Recording this increase in light transmission as a function of time, which in fact represents a decreasing salt concentration within the cuvette, yields the osmotic fragility curve. Electrodes in the outer distilled water bath can be used to record changes in conductance, and these can be translated into osmolarity or salt concentration changes within the cuvette.

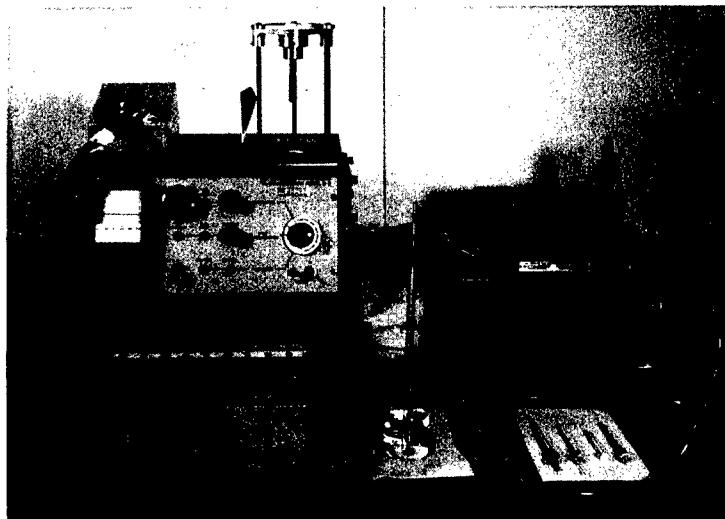


Figure 2. PHOTOGRAPH OF FRAGILIGRAPH MODIFICATIONS.

Figure 2 is a photograph showing some modifications in the fragiligraph as we employ it in our laboratory. First, we have devised a slide-type of elevator which allows us to introduce the cuvette into the instrument precisely into the light path. Therefore, the initial, rapidly changing portion of the fragiligraph is accurately obtained. Secondly, we have replaced the potentiometer used to set the 100% hemolysis mark with an equivalent ten turn potentiometer with a thousand point dial. Thirdly, with an attachment which can be purchased, we record fragiligraphs on an eleven inch Westronics Multipoint recorder rather than on the two and one quarter inch Rustrak normally supplied.

A further word on technique. In figure 3 some important points are demonstrated.

1. We obtain micro-hematocrits on each sample as this must be plugged into the computer.
2. We prepare a hemolyzed sample each time to set the 100% hemolysis mark as there is some drift and noise in the machine.
3. We use buffered saline which is iso-osmotic with 0.9% NaCl to dilute the blood so that the salt curve we obtain is an accurate representation.
4. All fragiligraphs are obtained at 37.5 C.

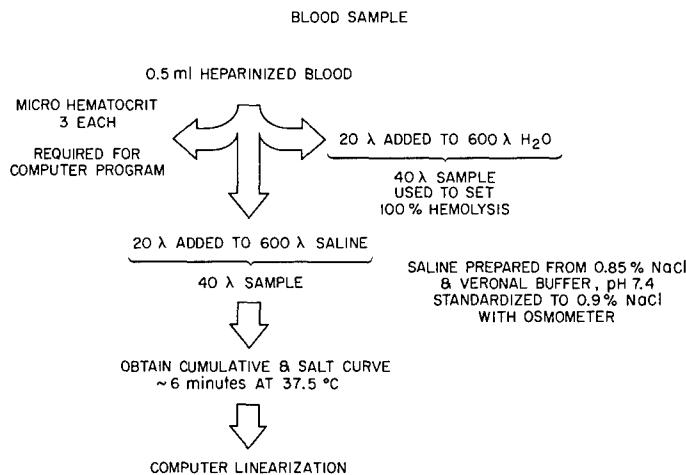


Figure 3. SAMPLE PREPARATION

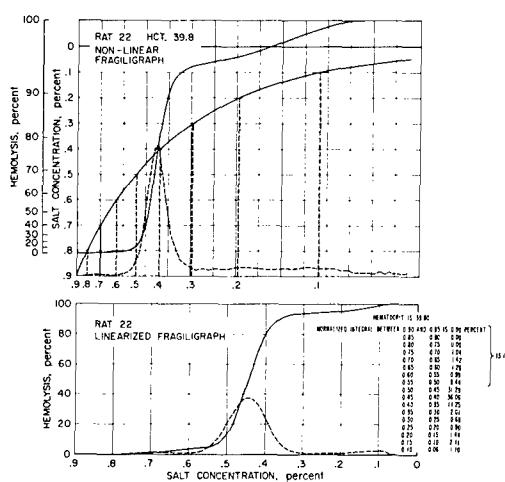


Figure 4. COMPARISON OF NON-LINEARIZED AND COMPUTER-LINEARIZED FRAGILIGRAPH.

A typical fragiligraph is illustrated in upper figure 4. Below, for comparison, are the same data after linearization using our computer program. Also inserted are data we obtain on a separate computer readout. Referring to the upper fragiligraph, I would like to point out the drawbacks which are, in fact, drawbacks in the mode of presentation rather than in the performance or sensitivity of the apparatus.

1. Percent hemolysis, on the ordinate, is presented in a non-linear fashion, the interval between 90 and 100% hemolysis being on the order of 15 times larger than the 0-10% hemolysis interval. In addition, the scale varies according to the hematocrit of the original blood sample.
2. The salt concentration changes, and is also recorded in a non-linear fashion. This is inherent in the design of the machine.
3. The non-linearity of the salt concentration curve consists of a compression of the early portion of the fragilogram. This portion presents the older or more fragile cells. Therefore, initial damaging exposures to toxicants which would most likely cause changes in these cells first would go undetected under present modes of presentation.
4. Exact comparisons are further complicated by the fact that the salt concentration curve, although generally the same, is hardly ever superimposable from one run to the next. This is due to changing membrane characteristics as well as to perturbations in the chart drive mechanism.
5. Both the percent hemolysis and salt concentration scales are initially on the same axis, as the salt calibration is done on the y-axis also. It is then transposed to the x-axis by coming off the salt curve for the particular run.
6. It has been demonstrated that the fragility values obtained with this instrument are generally similar to those obtained with the standard multiple tube technique. However, the non-linearity of the scales offered by the fragilograph makes a comparison to standard osmotic fragility curves difficult at best.
7. An electronically obtained derivative curve can be obtained with the fragilograph. However, it is distorted to the same extent as the cumulative percent hemolysis curve, and therefore quantification of the area under the curve is meaningless.

The corrective procedures we have applied via the computer are as follows:

1. The percent hemolysis scale, the y-axis, has been linearized. Using empirically obtained scales supplied with the instrument, a scale is chosen corresponding to the hematocrit of the initial sample.
2. The salt concentration changes are plotted linearly on the x-axis.
3. A linear cross plot of percent hemolysis and salt concentration is thus brought about.
4. The cumulative curve so linearized is differentiated by a subroutine and the derivative curve is plotted.

5. The linearized derivative curve is normalized and the percent of cells hemolyzed between each 0.05% salt interval is listed on a separate computer readout. These can then be easily summated, for example in this case we have 13.15% of the cells hemolyzing in up to 0.50% salt.

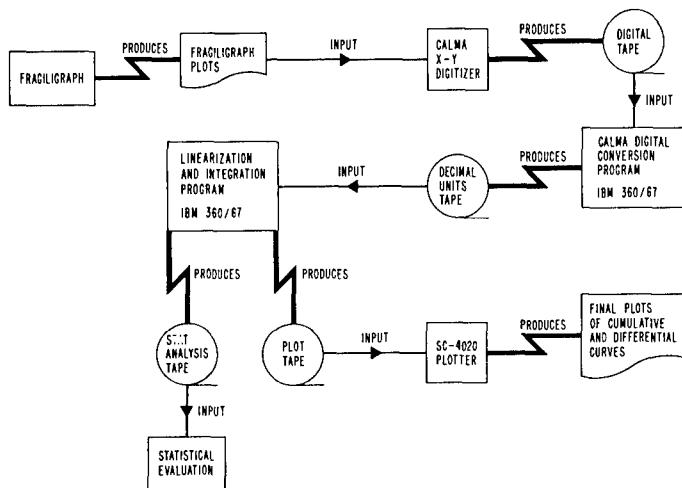


Figure 5. FRAGILIGRAPH DATA ANALYSIS SYSTEM.

The actual fragiligraph data analysis system is shown in figure 5. Thus, from the fragiligraph we obtain the non-linear fragiligraph plot. We must obtain the salt curve, the hematocrit, and the cumulative fragiligraph. From these continuous or analog recordings we obtain digitized x-y data using a CALMA x-y digitizer. This step involves a human operator physically tracing the curves and therefore is subject to noise depending on the ability of the digitizer operator. The digitized data are decimalized and linearized and integrated successively in the IBM 360/67. From this, two tapes are produced, one to be used by the statistician for further statistical analysis, and a tape for the plotter. The latter tape is fed into the SC 4020 plotter, and final curves are drawn of the cumulative and differential curves.

I would like now to show some comparative data and, hopefully, demonstrate the power of these combined techniques. In figure 6 are plotted non-linear fragiligraphs of a normal rat and one exposed to oxygen at 760 torr for 3 days. It is evident that there is some difference; namely, a shift to the left indicating that most if not all cells had become more fragile, indicating a damaging effect of the oxygen. In this case, the salt curves were unusually close, facilitating a comparison. Nevertheless, the more fragile, quicker responding portion is obscured by the discrepancy in hematocrits which accounts for the different starting levels as well as by the exponential scale compression. Attempts to quantitate such relationships have usually involved the establishment of arbitrary scales such as the point of 50% hemolysis.

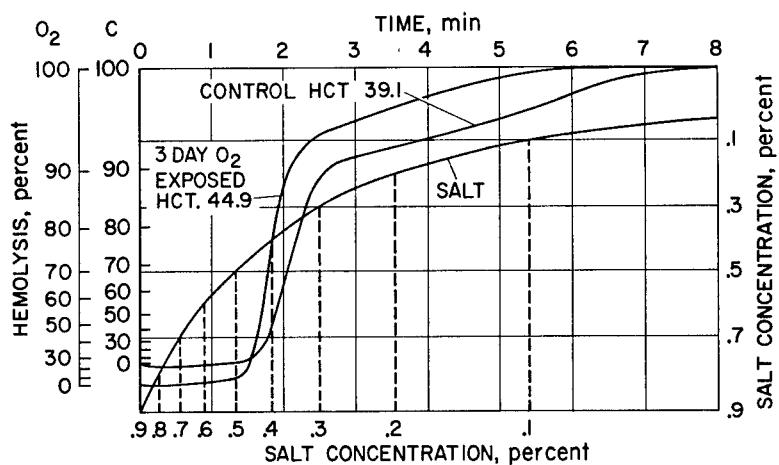
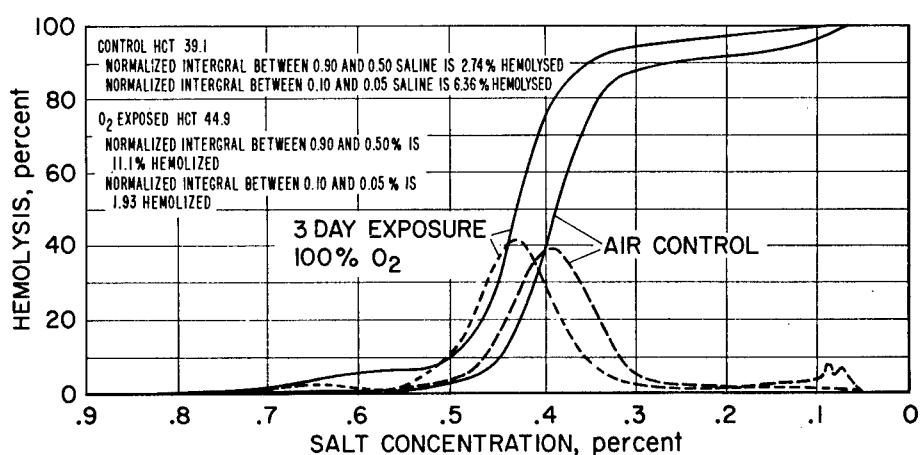
Figure 6. NON-LINEAR FRAGILIGRAPH OF A NORMAL AND  $O_2$  EXPOSED RAT.Figure 7. LINEARIZED FRAGILIGRAPH OF A NORMAL AND  $O_2$  EXPOSED RAT.

Figure 7 shows the same data linearized. Derivative curves and normalized data are also included. Data presented in this manner can be evaluated visually with ease, and a number of points of comparison are readily obvious. For example, although oxygen shifts the whole curve to the left, there appears to be a disproportionate increase in the more fragile portion of the population. A number of other points of comparison can be chosen depending on the nature of the exposure or experimental procedure.

In this series of test runs, part of which is illustrated in figure 7, we made a number of comparisons for exposure times varying from 3 hours to 3 days. From past experience we expected the older, more fragile cells to show the first effects, and therefore this time study was done to test the computer technique and to see if progressive changes could be detected. Statistical numbers were employed, and therefore individual curve comparisons are not appropriate. However, some of the data obtained from this series of experiments are listed in table I. This shows the statistical significance of the cumulative percent hemolysis comparing O<sub>2</sub> exposed rats to air controls for various periods of exposure and in two salt concentration ranges. The 0.55 and 0.50% range involves the older, more fragile cells and the 0.40 and 0.35% range, although consisting of older cells, is more representative of the average population and would constitute the ascending portion of the S-shaped curve. In this group it can be seen that significant differences can be demonstrated after 3 days of exposure, particularly in the 0.35% inclusive range.

SIGNIFICANCE OF PERCENT HEMOLYSIS FOLLOWING  
EXPOSURE TO 760 TORR O<sub>2</sub>

% SALT	AIR CONTROL	3 hr	P	1 DAY	P	3 DAY	P
0.55	2.4	6.1	<.05	7.9	<.002	6.0	<.02
0.50	5.1	10.4	<.02	11.4	<.01	9.9	<.01
0.40	44.7	47.2	N.S.	46.3	N.S.	64.8	<.10
0.35	71.3	74.5	N.S.	72.7	N.S.	89.5	<.01

On the other hand, if we examine the range of the older, more fragile cells, we see that oxygen at 760 torr can cause significant changes within 3 hours of exposure. This is due not to the magnitude of the change, but to the consistency. To me, the demonstration of this early change which I do not believe has been previously reported but has been surmised, demonstrates the extreme sensitivity of this combined technique. This is particularly true since the more significant changes are occurring in what amounts to a small fraction of the total population.

As a final demonstration of the utility of this technique, I would like to show you the comparison illustrated in figure 8. Here we compared normal rats kept in air and those kept in our exposure capsules for 2 days without the usual 24 hour cleaning. This was done with statistical numbers, but only representative curves are illustrated. It can be seen that even such conditions which may be considered a mild exposure can cause shifts in the linearized osmotic fragility curve which are just as severe as those seen with 3 days exposure to oxygen.

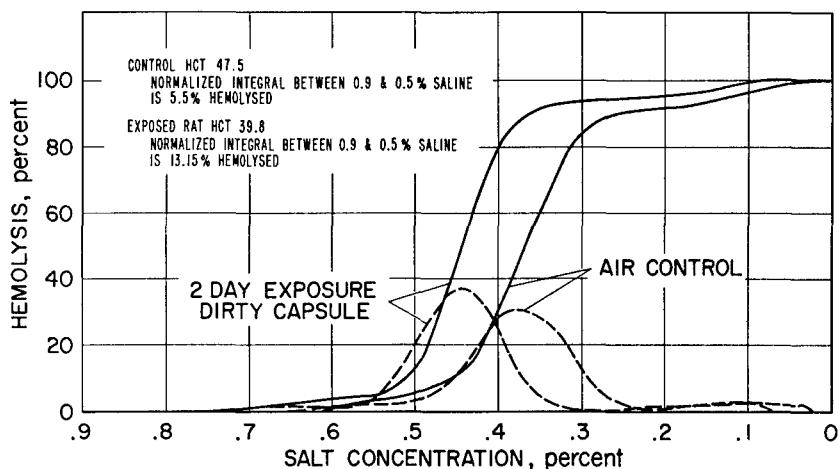


Figure 8. LINEARIZED FRAGILIGRAPH - CONTROL RAT VS. RAT IN DIRTY CAPSULE.

In conclusion, I would like to say that we have standardized our techniques for obtaining osmotic fragility curves of red blood cells using the Danon Fragiligraph, and using computer techniques have linearized the data, thus in effect magnifying that portion of the curve relating to the older or more fragile cells. This has allowed us to detect changes in fragility which would not be observable or readily quantified by other techniques.

REFERENCES

1. Danon, D.; "A Rapid Micromethod for Recording Red Cell Osmotic Fragility By Continuous Decrease of Salt Concentration"; J. Clin. Path., 16:377, 1963.
2. The fragiligraph is marketed by Kalmedic Instruments, Inc., 425 Park Avenue, New York, New York 10022.

## DISCUSSION

DR. THOMAS (Aerospace Medical Research Laboratory): Dr. Leon, is the program available?

DR. LEON (NASA, Ames Research Center): Yes, I have 10 copies of it in my briefcase if anyone wants it, you're welcome to it. I imagine it costs a few hundred dollars, but I didn't find out how much it costs.

MR. TOLIVER (Aerospace Medical Research Laboratory): Let me ask you first one question and then a second question. The first question is what were the increments of salt solution added by the fragiligraph?

DR. LEON: Oh, that is a continuous addition, if you were a computer man I suppose you would say it's an analog addition rather than a digital addition. Dialysis is a continuous process, so actually that is the beauty of the whole machine in that it doesn't do it in discrete concentrations but by continuously changing concentrations.

MR. TOLIVER: I am somewhat confused on this. May I use the blackboard?

MR. WANDS: Yes.

MR. TOLIVER: Unfortunately we started this by our fragiligraph breaking down. It is a heck of a way to start and we had several problems and we tried various methods of plotting to make it meaningful. I would certainly like to know what certain portions of the curve are. And as I talked with you at the beginning of the hour, we can consider the fragiligraph is a regular "S" shaped curve, put concentrations salt down on this axis and fragility on this axis. And just arbitrarily for discussion, let's divide the curve A, B, C, D, and E. As an old analytical chemist the thing that comes right to my mind-- this is a standard titration curve and means certain things to me at least those portions AB, BC, DE mean certain things. Now, are you able to tell me on the fragiligraph what these various portions of the curve really mean, what happens when the slope changes, what happens when the curve shifts?

DR. LEON: Well, that was the whole purpose of linearizing it, hopefully, but I look at it as a physiologist not as a chemist. The part from A to B represents normally the older cells which are the more fragile cells. Now if some toxic exposure makes the cells more fragile, it might affect all of the red blood cells equally in which case the whole curve would shift to the left or it might selectively affect older cells which

seems to be the case with oxygen in which case you might not see a shift in the whole curve but you might see an elevation in the segment from A to B. Upon getting the derivative of it there would be more area under the curve than the section from B to C. The point C is the inflection point and it does have some meaning ordinarily. If this were a perfect curve it would be the average of all the red blood cells, but I don't think the fragility curve is a perfect Gaussian curve so it doesn't have that much meaning. The section D to E represents the very young cells, they're not the reticulocytes and so we can tend to get a quantitation of erythropoiesis. There is a part that you don't show here, beyond E there would be another hump which when you integrate it, when you get the first derivative, you would form a separate little peak, let me show you, the curve X is, of course, the first derivative of the major "S" shaped curve, that little hump past E actually represents the reticulocytes, they form a different population and at the moment we've been trying to see if we can't quantitate reticulocytes by the size of that secondary peak. We get qualitative relationships, but we can say if it is big there is a lot of reticulocytes and if it's little there's not too many, but other than that I don't know that we'll be able to say how accurate we'll be able to get these answers. Has that answered your question?

MR. TOLIVER: That helps me considerably, and let me ask you one more question, if I may. Did you attempt to do a semilog plot with these in an attempt to expand your scale?

DR. LEON: I've heard of that technique but I haven't tried it, no.

ANALYSIS OF SPONTANEOUS ACTIVITY FILMS  
BY A FULLY AUTOMATED ANALYZER

Anthony A. Thomas, M. D.

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio  
and  
Barrett L. Myers

Technology Incorporated  
Dayton, Ohio

INTRODUCTION

Space cabin toxicology experiments conducted by the Air Force Aerospace Medical Research Laboratories concluded that spontaneous activity of unrestricted dogs measured by time lapse photography would furnish good behavioral information when properly analyzed. Several hundred hours of photographic data were obtained and a large amount was analyzed using visual and rudimentary electronic techniques. Due to the time consuming task of analyzing the large amount of data, we theorized that an electronic system could be developed which would automatically categorize and display the change in spontaneous activities and further refine the experimental and analysis techniques used to analyze the time lapse photographic data. This approach should result in a system which would completely eliminate the photographic process and monitor the animals in real-time using closed circuit television or a special camera.

METHODS

The changes in spontaneous activity are optically monitored and categorized into nine graded activity levels. Each level of spontaneous activity has a counter which counts and adds the occurrence of each monitored level of change. Consequently, the animals can be monitored with normal ambient light for a period of 24 hours and the resultant data printed and plotted for operator analysis. The analyzer was designed to analyze in three modes of operation: time lapse photography, live closed circuit television displays or live through a ground glass camera.

The instrument monitors and categorizes the animals' spontaneous activity by optically monitoring two areas in the experimental environment, the reference area and the activity area. The reference area is an area in the optical presentation where no activity can occur and provides a general background light reference level. Normal ambient light is adequate. The activity area is the area where the animals are free to move. When the animals move, the average reflected light intensity of the activity area changes relative to the reference area which remains constant. These two areas are monitored by one each separate light sensitive device which provides an electrical output proportional to the average reflected light intensity. The optical detectors convert the average reflected light intensity to an electrical signal which is electronically conditioned before the reference value is subtracted from the activity value. The resultant signal which is representative of a particular activity is then stored and compared with the next observation signal, and, when a difference exists indicating a change in the presence or absence of spontaneous activity, the difference is measured by categorizing it into levels. This is accomplished by comparing the resulting voltage with the voltages that are assigned to the activity levels #1 through #9. After the highest level is matched it is summed with the previous counts at that level.

Two forms of display are provided for the operator. The first form is an analog plot wherein the activity level is plotted versus the number of activities that occurred in that level. The total number of activity changes without regard to the particular levels and the weighted average of the 9 activity levels is plotted in analog form. The second form of display is a tabulated printout which is comprised of a numerical listing of the number of changes in spontaneous activities at each activity level as well as the sum of the total number of activity level changes.

The analyzer consists of five major subunits which include two optical detectors, the control unit, the X-Y plotter and a printer (figure 1). The optical detectors are connected to adjustable stands to facilitate positioning them in the reference and activity areas. The primary operator control features of the system are located on the control unit. From left to right on the control panel are the power switches including system power, printer power and plotter power. There is a pushbutton switch which causes a printout. A printout does not destroy the stored data. The switch under the meter selects which sensor's electrical output the meter will be monitoring. The meter is also required in order to adjust and calibrate the overall sensitivity of the system. As shown, the meter can monitor either the output of the activity area photo sensor or reference area sensor, or the difference signal between the reference and activity sensors. The gain of the entire system can be adjusted to optimize all three modes of operation. In order to accomplish the three modes of operation (time lapse, live TV and camera), special sampling provisions were included which allow the operator to select the mode of operation manually. These sampling modes included internal and external synchronization so that either live presentation (television or camera) could be monitored. In the internal mode the sampling rate is 60/min, 30/min, 12/min, 6/min or 1/min. In the film mode the analyzer samples the inputs shortly after the projector chopper clears the projection window.

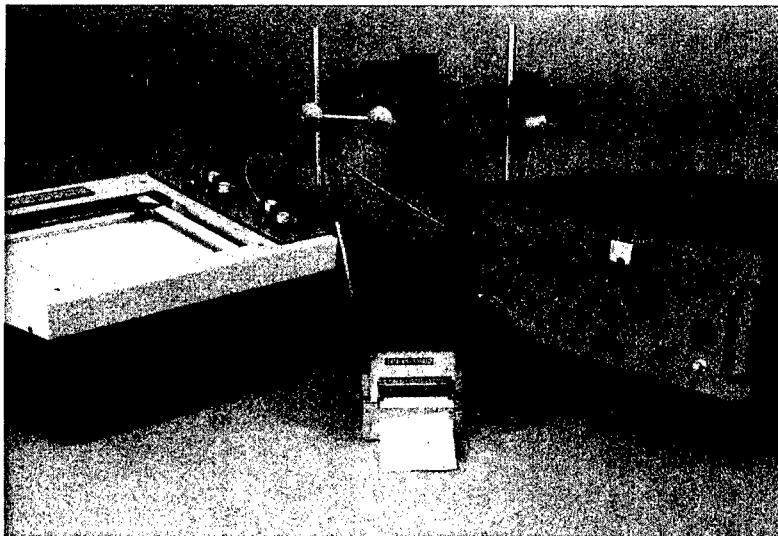


Figure 1

The photo detectors are housed so as to reduce the effects from ambient light and also to accomplish focal point adjustment. Accommodations for mounting glass lenses have been provided on the front of the tubes. The glass lenses could provide focusing or defocusing capabilities.

All computations are accomplished in the control unit and include determining the changes of the intensity of light from the activity area with respect to the reference area, measuring the magnitude of the change, and logically categorizing it into one of nine different activity levels.

Figure 2 shows a block diagram of the spontaneous activity analyzer. In general the outputs of the light sensitive photo transistors are amplified, subtracted, sampled and stored and then compared with the next subtracted output. When a difference exists it is categorized, advances the appropriate integrated circuit counter, and is stored for printout or plotting upon operator command.

The two blocks entitled Ref. and Det. represent the reference area optical detector and the activity area optical detector.

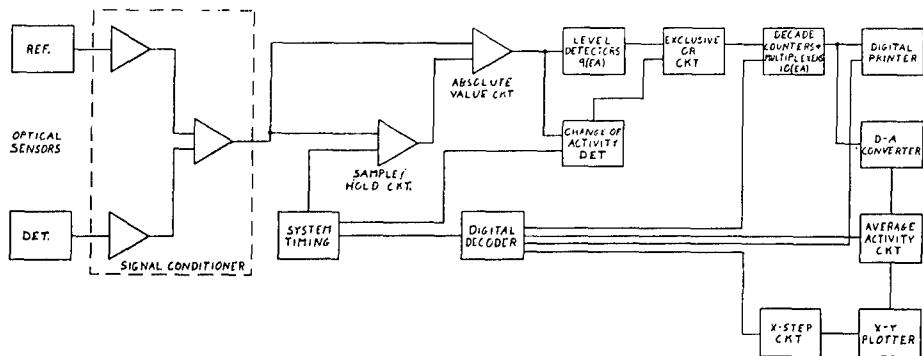


Figure 2. SYSTEM BLOCK DIAGRAM  
SPONTANEOUS ACTIVITY  
ANALYZER

The three triangles located in the dotted square represent operational amplifiers and comprise the signal conditioning. The photo transistors could not be coupled directly with the subtraction circuit, so two operational amplifiers with special characteristics were employed to interface with the detectors. These two amplifiers are represented by the two triangles on the left. The outputs of these two amplifiers are connected to the subtraction amplifier.

The third op. amp. actually subtracts the reference area signal from the activity area signal with the remainder being the light reflected by the experimental subjects. This remainder is applied to the sample and hold circuit. The sample and hold circuit is commanded to sample the remainder from the subtractor circuit and to hold that value until commanded to sample again. Before the next sample but after a new remainder has been derived by the subtractor circuit, the value contained in the sample and hold circuit is compared with the new remainder. This is accomplished in the absolute value circuit. Since decreases in spontaneous activity are frequently present, this subtraction process could result in negative values and consequently a circuit was employed which would subtract the difference between the two inputs but always provide a positive value out. That is, the output is always the absolute value of the difference between the two inputs whether positive or negative in nature. The change in activity detector determines if a change has occurred and if the magnitude is at least one activity level. If this is not the case, nothing happens. If the change is at least one level, the output is allowed to be applied to the Exclusive OR circuits. The nine level detectors respond to the highest level of activity that has been surpassed. These outputs are applied to exclusive OR circuits, which insure that only the highest level is allowed to be counted by its counter. The decade counters are comprised of ten counters, each capable of counting to 99,999. These counters total the occurrence of individual activity

levels. The system timing provides timing signals through an internal clock which instructs the various elements when to perform their functions.

When the operator depresses the print and plot button on the front panel of the analyzer, the contents of each digital counter are applied to the printer and digital-to-analog converter. The printer simply tabulates the contents of the counters, but the digital-to-analog converter actually converts the digital signals to their analog equivalent for plotting on the X-Y recorder. The X step circuit steps the X-Y plotter to the right each time another level is recorded, and the average activity circuit computes the weighted average of spontaneous activity.

## RESULTS

The actual presentation of the spontaneous activity as displayed on the X-Y recorder is illustrated in figure 3. This is a record of the spontaneous activity of four beagle dogs. The various activity levels are seen displayed from left to right, beginning with activity level #1 which is the lowest step. The following steps are steps 2 through 9, with #9 being the highest level of activity. The largest excursion on the record is the total activity which is the sum of steps 1 through 9. The last excursion is the weighted average activity level for all steps which the instrument automatically calculates. This recording represents a 1-hour real-time period which was recorded by time-lapse photography technique, where each frame was taken at 30-second intervals.

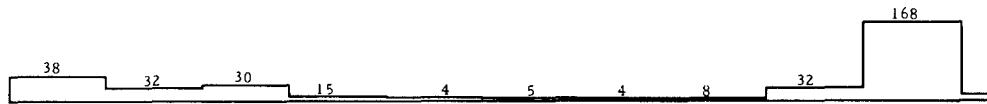


Figure 3

Figure 4 illustrates the resolution that can be achieved on the same time lapse film strip by projecting individual sections of the film, representing 2-hour real-time periods. In this instance, only total activity was plotted against real-time. The beginning of the record is at 0945 and the end is about the same time on the next morning. One can readily see that total activity had an occasional peak between 1500 and 1600 when the animals were fed and was fairly constant during daytime, but dropped off markedly in the late night period.

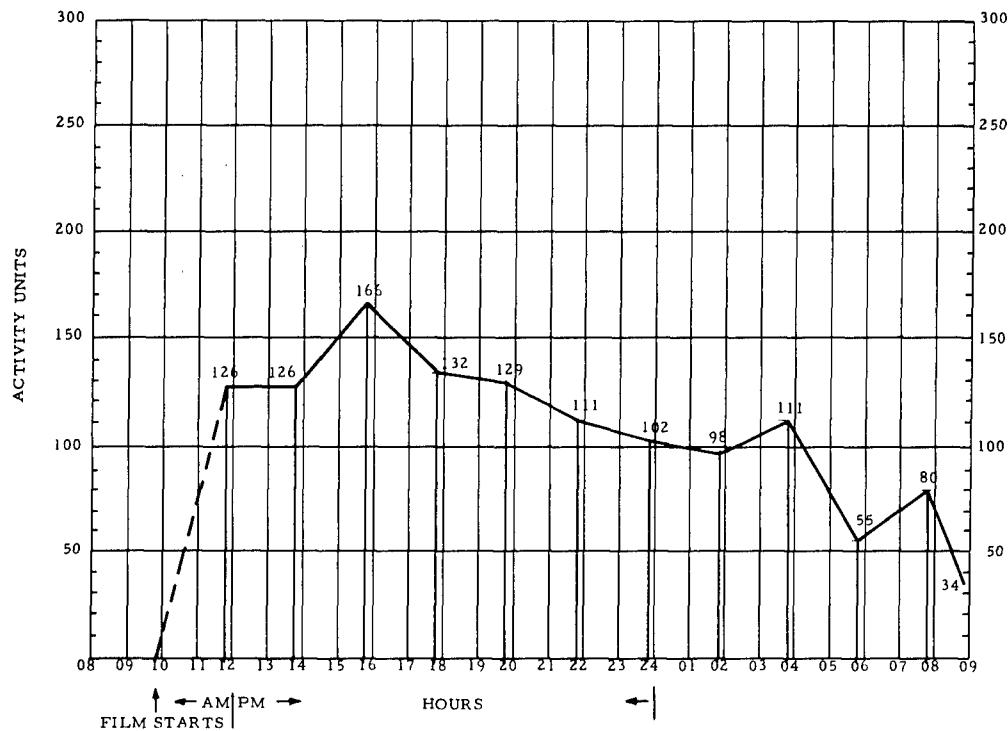


Figure 4. TOTAL ACTIVITY IN EACH 2 HOUR PERIOD

To illustrate the actual use of the time lapse photography technique, data from a recent continuous exposure study with Freon 113 (fluorocarbon 113) are presented. This compound is one of the atmospheric contaminants identified by the Panel on Air Standards for Manned Space Flight (Space Science Board, National Academy of Sciences) as an ubiquitous contaminant of significant concern in life support simulators and in some Gemini and Apollo flights. It is a solvent used in the manufacturing and assembly process of the command module and associated life support systems. The provisional tolerance limits recommended by the Panel were 200 parts per million (ppm) for 60 minute emergency exposures and 20 ppm for a 90 day mission. No limit could be recommended for 1000 day mission durations because of the lack of good chronic exposure data.

Freon 113 was shown to cause decrement in psychomotor performance in human volunteers at 2500 ppm for two hours. Subjective symptoms included sleepiness and a feeling of heaviness in the head (Stopps and McLaughlin, 1967).

The TLV for an 8 hour daily occupational exposure is 1000 ppm, but U. S. Army investigators noted marked subjective drowsiness in employees working in explosive assembly plants, where their survey showed Freon 113 concentrations to be generally below 1000 ppm with occasional peaks of 2000 to 3000 ppm (U. S. Army Environmental Agency Reports on Projects 23-25-68; 32-28-68; 32-43-68).

In our exposure studies with Freon 113 we monitored around-the-clock the spontaneous activity of unrestrained dogs for the entire two week continuous exposure to 2000 ppm. For the sake of brevity, only data from two 24 hour periods of time lapse film are presented, the second day and the last complete day (24 hours) of exposure (figures 5 and 6) as displayed on the x-y recorder.

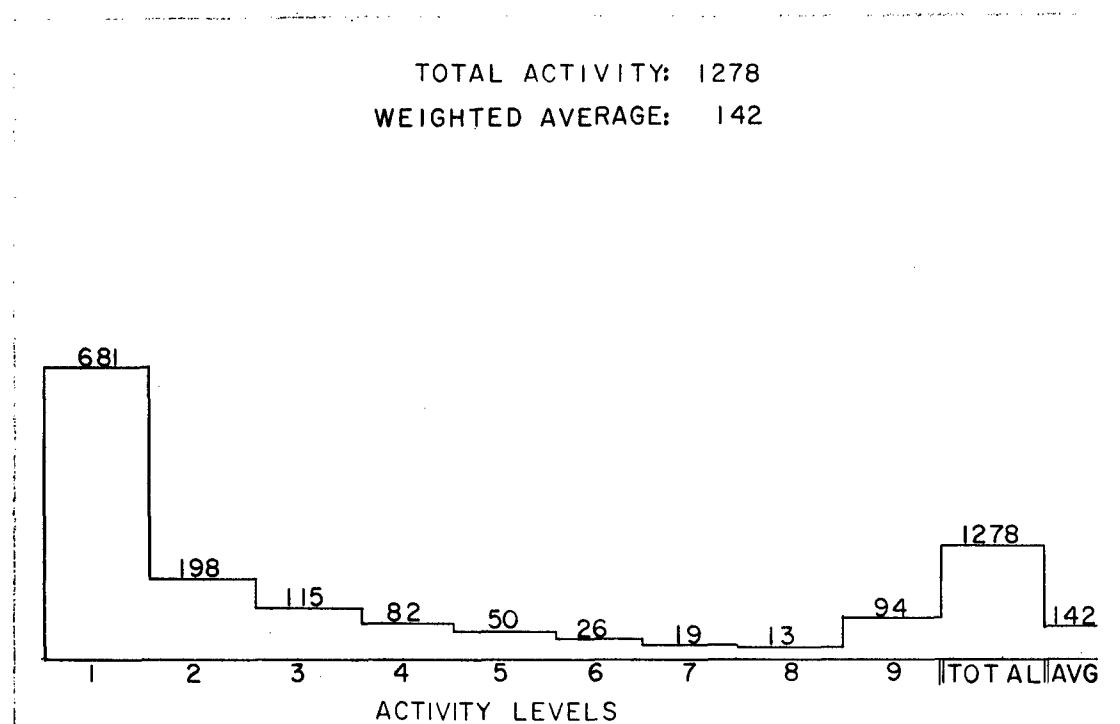


Figure 5. SPONTANEOUS DOG ACTIVITY.  
Second 24-hour period of Freon  
113 exposure (2000 ppm).

TOTAL ACTIVITY: 1066  
 WEIGHTED AVERAGE: 118

CHANGE FROM 2<sup>ND</sup> DAY

TOTAL ACTIVITY DOWN BY 212  
 WEIGHTED AVERAGE DOWN BY 34

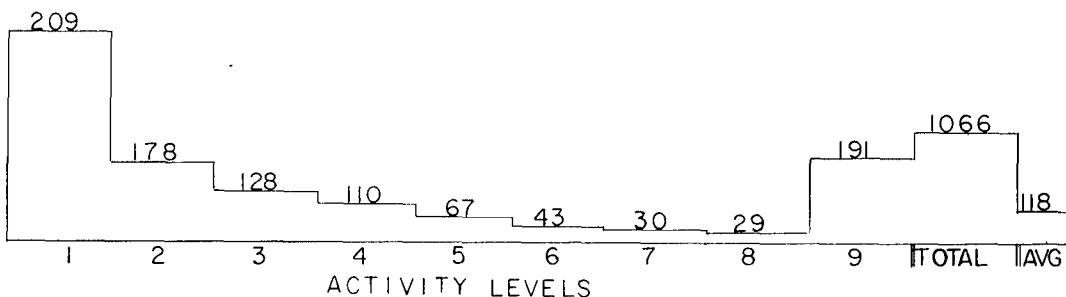


Figure 6. SPONTANEOUS DOG ACTIVITY.  
 13th 24-hour period of Freon 113  
 exposure (2000 ppm).

There are some obvious changes between the beginning and the end of the exposure which are tabulated in table I. Since the accuracy of counts by the Spontaneous Activity Analyzer is within  $\pm 5\%$  of each true value at any activity level, including the total activity, only those values where the difference is significant in terms of increased or decreased activity have been marked.

#### DISCUSSION

The combination of digital counting techniques with an analog display on the x-y recorder represents a great improvement in the evaluation and interpretation of spontaneous activity as a behavioral test method.

TABLE I  
SPONTANEOUS DOG ACTIVITY  
2000 PPM FREON EXPOSURE

<u>ACTIVITY LEVELS</u>	<u>2nd 24-HR PERIOD</u>	<u>13th 24-HR PERIOD</u>	<u>CHANGE</u>
1	681	290	Signif. ↓↓↓
2	198	178	N.S.
3	115	128	N.S.
4	82	110	Signif. ↑
5	50	67	Signif. ↑
6	26	43	Signif. ↑↑
7	19	30	Signif. ↑↑
8	13	29	Signif. ↑↑
9	94	191	Signif. ↑↑↑
 TOTAL	 1278	 1066	 Signif. ↓↓
WTD. AVG.	142	118	Signif. ↓

Since the Spontaneous Activity Analyzer is essentially a real-time data logger, the resolution and speed of the process have been tremendously increased over the "length of trace" method previously described (Thomas, 1967).

In interpreting the results from the two week continuous exposure of dogs to Freon 113, there is no question of the significance of the decreased spontaneous motor activity at the first activity level (small movements) and at the total and weighted average activity levels. This depression would indicate a slight sedation of the dogs due to the Freon exposure and tends to support the previous human exposure experience. The increasing activity trend from activity level 4 up to 9 is also real and may well indicate a cyclic phenomenon not uncommon with central nervous system irritants where periods of depression are interrupted by brief hyperactive episodes. At this time we cannot prove the validity of this explanation but further experiments with drugs using mice are scheduled in order to establish the true degree of resolution of which this method is capable.

By using live closed circuit television pickup and a clock operated command for repetitive printout by the digital printer (which does not destroy the accumulated counts), one can sample from the TV screen at one second intervals and break down the history of spontaneous activity into 5 minute episodes. This increase in resolution (86,400 observations during a 24 hour period, broken down into 288 consecutive 5 minute episodes) should be capable of accurately detecting hyperactive or convulsive periods as well as sedative and hypnotic effects. Both time of occurrence and duration of effect thus can be documented to the nearest 5 minute sampling period.

With all these functions accomplished automatically, the savings in skilled observer time can become quite lucrative. We all remember the endless waiting periods one spends in trying to catch the occurrence of delayed convulsive episodes in drug studies. We also believe that by using a closed circuit television system and placing the reference and detector sensors on the monitoring screen, one could economically increase the resolution of spontaneous activity to the point where it may be able to detect circadian rhythms if they are reflected by spontaneous activity. The number of observations is limited only by the TV scanning frequency and cycling of the digital printout device.

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## DISCUSSION

DR. INGRAM: I didn't understand exactly what your photometric measurement was. Is this reflected light from the animals?

DR. THOMAS: Yes, this is done on actual photographic pictures. The problem with the other techniques is when you either interrupt a light beam, or something like that, that you don't really quantitate the amount of activity. With this method you can detect if a Beagle dog turns over showing more white area on his belly so you have tremendous resolution.

## PERMEATION TUBES AS CALIBRATED SOURCES OF GAS

Bernard E. Saltzman, Ph. D.

University of Cincinnati  
Cincinnati, Ohio

Known low concentrations of gases are needed for toxicological studies, for calibration of instruments and for validation of analytical methods. A variety of techniques that may be used for their preparation have been outlined by Saltzman (1961) and Hersch (1969). More recently permeation tubes were developed by O'Keeffe and Ortman (1966) as convenient sources. Liquefied gases such as sulfur dioxide, nitrogen dioxide, hydrocarbons and hydrogen sulfide were sealed in Teflon tubing and were found to permeate through the walls for long periods at relatively constant rates of a few milligrams per day. Such tubes also were used successfully for anhydrous ammonia (Scaringelli et al, 1970), anhydrous hydrogen fluoride (Elfers et al, 1968; Jacobson, 1967), phosgene and organic mercury compounds (Linch et al, 1968) and for hydrogen sulfide and mercaptans (Bamesberger et al, 1969). A critical study of sulfur dioxide permeation tubes was made by Scaringelli et al (1967). These tubes also were successfully used by Thomas et al (1966) and collaboratively tested by the National Air Pollution Control Administration (Saltzman, 1961; Tye et al, 1968). Permeation tubes have been found to be simple, convenient and relatively constant sources for periods as long as a year. Although they have been applied mainly for calibration of instruments, by appropriate modifications they should be suitable for toxicological chamber work.

### Construction of Permeation Tubes

Although any inert plastic may be used, Teflon tubing has been preferred for manufacture of permeation tubes because of its chemical inertness and stable mechanical properties. Both FEP (fluorinated ethylene propylene copolymer) and TFE (tetra-fluoroethylene polymer) Teflon have been used. The former is considerably less porous and provides lower permeation rates than the latter. Sizes that have been commonly used are 1/4" O.D., 0.030" wall thickness and 1/8" O.D., 0.030" wall thickness. Other dimensions may be used in order to contain the pressures and to adjust the permeation rates and lifetimes to those desired.

Originally the tube ends were sealed by ramming in glass or stainless steel balls (O'Keeffe, 1966). The liquefied gas was introduced from a cylinder connected to a

sealed permeation tube by pinching the tubing to open a passageway around the ball at the end connected to the cylinder. If necessary, cooling with ice was used to promote the filling process. Entrapped air was vented, if desired, by disconnecting the partially filled tube from the cylinder and pinching again around the ball. The tube was then reconnected to the cylinder and filled. After this was accomplished, the ball was rammed deeper into the tube to a portion not damaged by the pinching.

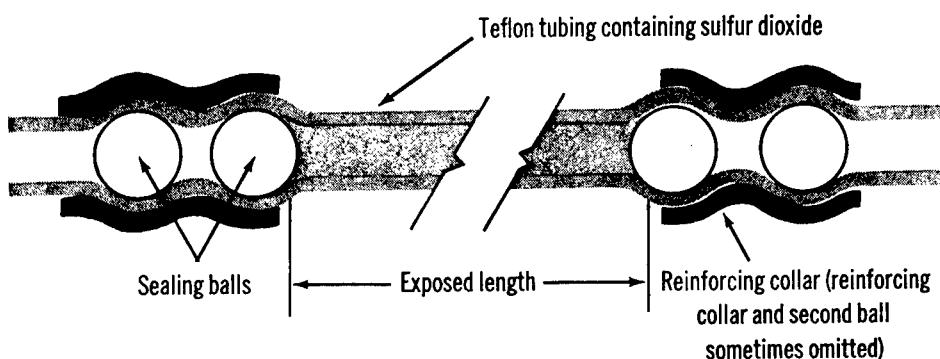


Figure 1. CONSTRUCTION OF SULFUR DIOXIDE PERMEATION TUBE.

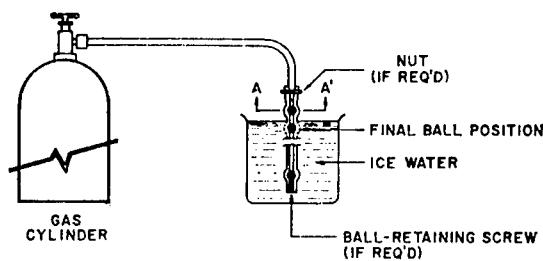


Figure 2. PROCEDURE FOR FILLING PERMEATION TUBES.

Another technique used was to fill the tube at atmospheric pressure by cooling it below the liquefaction point of the gas with ordinary or with dry ice. Various other methods have been employed for sealing of tubes. The seal is obviously critical, since any leakage destroys the reproducibility of gaseous emission. Commercial tubes are available with ends plugged with solid Teflon rod, held in place with crimped bands of stainless steel or aluminum. Alternatively, standard 1/4" Swagelok ferrules have been used as crimped bands; fused ends also have been used (Saltzman et al, 1970).

The lifetime of a tube is determined by the permeation rate and the weight of its content of liquefied gas. For a given plastic tubing, the lifetime is proportional to its volume to surface ratio and also to its wall thickness. Because an appreciable amount of time can be consumed during the calibration procedure, lifetimes of a year or more are highly desirable. These have been achieved by using heavy walled bottles drilled out from Teflon rod (Scaringelli et al, in press) or by attaching glass or stainless steel bottles to the tubing (Saltzman et al, 1970).

The quantitative permeation relationships are theoretically as follows for a completely filled tube:

$$G = C \pi d/t \quad (1)$$

$$L = \frac{\pi d^2 \rho}{4G} = \frac{\rho dt}{4C} \quad (2)$$

$G$  = gravimetric rate,  $\mu\text{g}/\text{min}/\text{cm}$  of tube length

$C$  = permeation rate constant at temperature of use,  $\mu\text{g}/\text{cm}^2/\text{cm}$  thickness/min. This includes effect of the vapor pressure of the liquid.

$d$  = effective inside diameter of tube, cm

$t$  = wall thickness of tube, cm

$L$  = useful lifetime of tube, minutes

$\rho$  = density of liquified gas,  $\text{gm}/\text{cm}^3$

### Calibration of Permeation Tubes

After a new tube is manufactured, an equilibration period of as long as several weeks is required (Saltzman et al, 1970; Scaringelli et al, 1970) before a steady permeation rate is achieved. It is desirable to age the tube at a temperature above the ordinary range through which it will be exposed, such as at 30 C. For some substances, the contents are under appreciable pressure, and the tubes should be handled cautiously. They should not be subjected to excessive heat nor should they be scratched, bent, or mechanically abused. If nitrogen dioxide tubes are exposed to high humidity for a period even as brief as 15 minutes a visible whitening of the tube, possibly due to formation of nitric acid within Teflon, becomes apparent. This may cause a permanent decrease in the permeation rate. A similar problem may occur with other hygroscopic materials, such as anhydrous hydrogen fluoride. Exposure to oxygen also

has been noted (O'Keeffe et al, 1966) as affecting hydrogen sulfide tubes, probably by precipitating colloidal sulfur within the walls of the tube. The best type of storage for a tube is in a bottle submerged in a thermostated water bath, flushed with a slow stream of dry nitrogen from a cylinder.

Gravimetric calibrations may be made by weighing the tubes at intervals sufficiently long to obtain accurately measurable differences. The most effective way of determining the permeation rate is by plotting weight against time and fitting a line to the measured points. For an ordinary balance with the sensitivity of 0.1 milligram, this process may take as long as several weeks. The period can be shortened to a day if a good micro balance is available. For a corrosive gas, the permeation tube may be inserted in a tared stoppered glass tube or cylinder to protect the balance. Static charges, which develop on tubes such as those for propane (Saltzman et al, 1970; Scaringelli et al, 1970), can cause serious weighing errors. A polonium strip static eliminator is recommended to make accurate weighing possible.

Volumetric procedures (Saltzman et al, 1970; Saltzman et al, 1969) also may be used for the calibration. This technique has the advantage of rapidity. A microgasometer with a sensitivity of 0.2 microliters was used as shown in figure 3. Good calibrations could be obtained in less than an hour. Another advantage of this technique was that the tube could be calibrated *in situ* in the flow dilution system under the identical conditions and close in time to its actual use. For conducting a calibration, the permeation tube is contained in a test tube connected to the sensitive manometer, on the other side of which is a temperature compensating bulb. Dodecane has been used as a nonvolatile inert manometer fluid. The liquid meniscus is then restored to the line by withdrawing the plunger of a micrometer syringe which is in the apparatus on the same side of the manometer as the tube. This process is continued over a period of an hour or two. A plot of permeated gas volume vs time is made, the slope of which is the permeation rate.

The permeation process has an appreciable activation energy, which is the sum of the heat of vaporization of the liquefied gas and of the energy required to momentarily rupture interchain bonds in the plastic as the gas molecules force their way through the walls of the tube. This results in a rather high temperature coefficient, of the order of as much as 10% per degree C. Therefore, for 1% accuracy, temperature control to at least 0.1 C is needed. Equation 3 gives the relationship between permeation rate and temperature. It is of the usual Arrhenius form.

$$\log \frac{G_2}{G_1} = \frac{E}{2.303 R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad (3)$$

where  $G_1, G_2$  = gravimetric rates at different temperatures  
 $T_1, T_2$  = corresponding temperatures, K (= C + 273.16)  
 $E$  = activation energy of permeation process, cal/g mol  
 $R$  = gas constant, 1.9885 cal/g mol

In table I are listed some permeation rates for various substances in commercially available tubes. Lengths from 2 to 30 cm may be specified.

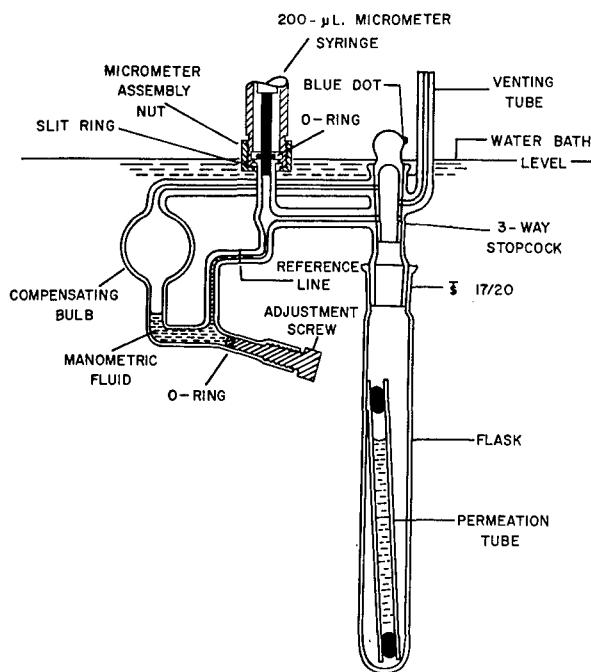


Figure 3. SCHEMATIC DIAGRAM OF MICROGASOMETER ASSEMBLY SUBMERGED IN WATER BATH. Apparatus, except for flask, commercially available as Gilmont Warburg compensated syringe manometer. Capacity of micrometer syringe, 200  $\mu$ l, in 20 turns with 0.2  $\mu$ l divisions. Stopcock, shown in venting position, is turned 180° for operation.

TABLE I  
PERMEATION RATES FOR SOME COMMERCIAL TUBES

	Permeation Rate, $\mu\text{g}/\text{min cm}$	
	Dynacel Tubes <sup>a</sup> at 22 C	A. I. D. Tubes <sup>b</sup> at 30 C
SO <sub>2</sub>	0.223	0.290
NO <sub>2</sub>	0.870	1.200
H <sub>2</sub> S	0.210	0.250
Cl <sub>2</sub>	1.25	1.50
NH <sub>3</sub>	0.280 (30 C)	0.170
Propane	0.040	0.080
n Butane		0.002
Propylene	0.240 (30 C)	
Isobutylene	0.014 (25 C)	
Methyl mercaptan	0.036 (30 C)	0.030

<sup>a</sup> Available from Metronics Associates, Inc., Palo Alto, California 94304  
Tubes are 3/16" O.D.

<sup>b</sup> Available from Analytical Instrument Development, Inc., West Chester, Pennsylvania 19380. Tubes are 0.062" wall thickness, except for methyl mercaptan, which is 0.030" wall thickness.

#### Use of Permeation Tubes

Permeation tubes are used under thermostated conditions in a flow system as shown in figure 4. This system may also be used for gravimetric calibrations. The small temperature fluctuations between heating and cooling cycles of the thermostated water bath do not disturb such calibrations.

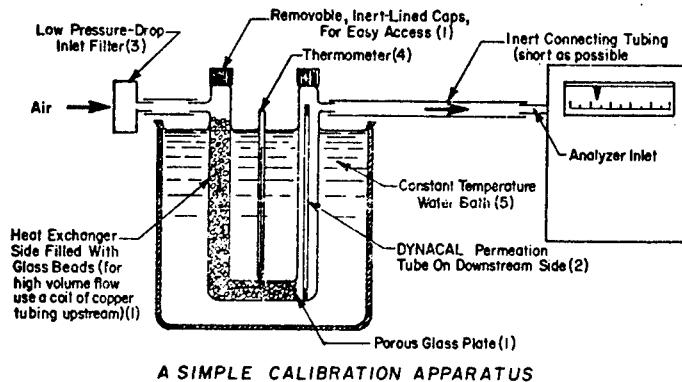


Figure 4. FLOW DILUTION SYSTEM FOR USE OF PERMEATION TUBES.  
This system is commercially available.

The microgasometer apparatus shown in figure 3 has also been adapted (Saltzman et al, 1970) for a flow system by adding a stopcock and gas inlet tube to the bottom of the flask. The venting tube is connected to a mixing bulb for dilution of the outlet gas with a metered flow of pure air. This arrangement permits rapid *in situ* calibration of the permeation tubes by shutting the stopcocks. For such volumetric calibration, however, a very stable temperature is needed. The thermostated water is therefore circulated through an aluminum coil immersed in an insulated bath which contains the microgasometer.

In both of these systems an inert metered gas stream flows past the thermostated permeation tube and is then blended with a larger flow of dilution air. The flow of the mixture should be exactly equal to or in excess of that needed, so as to avoid drawing outside air into the system. After the amount desired is drawn off from a side arm, the excess can be vented through a long tube.

Most applications have been for instrument calibration; however, with minor modifications and adjustments to produce the permeation rate required, the technique should serve equally well for exposure chambers. For some applications stable plastic materials other than those mentioned above may be suitable. The plastic should not contain any plasticizer, of course, since the latter would slowly bleed out and change the properties of the tube. It should be noted that permeation is a two way process. Care should be taken that undesirable materials such as moisture do not permeate into the tube over long periods. This technique has been adapted for gases which are not liquefiable by using a plastic disc in a suitable fitting connected directly to a gas cylinder. The latter then serves as an infinite reservoir providing constant pressure as the gas permeates through the disc at a very small rate. Reverse permeation is an important problem with this arrangement, also, that must be considered. In a good system, permeation tubes can provide very constant concentrations for long periods.

## SUMMARY

Permeation tubes have been recently developed as constant long lived sources of small quantities of gas. A liquified gas, such as sulfur dioxide, nitrogen dioxide, hydrogen sulfide, ammonia, hydrogen fluoride, and propane, is sealed in a Teflon tube. The gas permeates through the walls and escapes at the rate of a few milligrams a day, at a constant rate under thermostated conditions for periods of up to a year. The tube may be calibrated gravimetrically or volumetrically. These sources are convenient to use in a flow dilution system for calibrating instruments and may be adapted for use with exposure chambers.

## ACKNOWLEDGMENTS

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## DISCUSSION

COLONEL STEINBERG: Dr. Saltzman, did you say that permeation tubes were available for HF?

DR. SALTZMAN: They are not commercially available, but there have been reports in the literature. There are two papers listing anhydrous HF in permeation tubes and there are references in my paper which has been submitted here already. I could give you the references if you are interested.

COLONEL STEINBERG: I am, thank you.

DR. DOST: What about the problem of agents in the atmosphere moving into the tube, water for example, oxygen in some case, etc.?

DR. SALTZMAN: It should be emphasized that permeation goes in both directions and if you have a very hydroscopic material, you certainly should protect it from water vapor. Now, the only way of handling this, I think, is to maintain a relatively dry atmosphere and cylinder nitrogen is about as dry as is convenient to get. Keep it quite dry, and hope that you reach equilibration. Just have a slow flow and you always keep the tube in this same environment so that it reaches a steady state.

DR. DOST: This is a problem particularly with HF that was just mentioned, this is very difficult to keep dry.

DR. SALTZMAN: I think you just have to maintain it in a closed system, apparently it was used successfully in this way.

DR. MAC FARLAND: There was something about these permeation tubes that I wondered about. In your last slide where you showed an apparatus in which you can place the permeation tube and leave it undisturbed, but by a switching process you can change it to the calibration mode or supply mode. The assumption that this is based on is that if you have a flow of gas going past the permeation tube, this does not affect the rate at which the contents permeate out, and I've wondered about this assumption. To what extent does the concentration gradient outside the tube affect the rate of permeation? The assumption in this thing would be that it doesn't affect it at all.

DR. SALTZMAN: The permeation rate is proportional to the pressure difference inside and outside the tube. Now if you have an appreciable accumulation of gas on the outside, for example in a static container, eventually the pressure on the outside would equal that on the inside and the permeation process would stop. In the equation, you could put in a correction factor, but usually the factor is negligible because, for example if you have sulfur dioxide, the pressure on the inside is three atmospheres or three million parts per million and if you're running a few parts per million on the outside it's negligible. In the case of some other materials you could put in a mathematical correction. As far as the inert materials are concerned, the flow rate has no effect on the permeation rate whatsoever. The only thing that counts is the partial pressure of the permeating material. But as I mentioned, there are some disturbing effects, in some cases, for example from humidity and oxygen which must be controlled.

MR. TOLIVER: Was there any particular reason for using the permeation tube over an exponential dilution device?

DR. SALTZMAN: The virtue of a permeation tube is that you have a constant source with a lifetime of a year and assuming that you calibrate it and store it under good conditions, you know that you're getting so many milligrams a day coming off for a long period of time, and this is experimentally more convenient than an exponential dilution device.

MR. TOLIVER: I was a little bit disturbed by the fact that you said you had to maintain a temperature within a tenth of a degree Centigrade. This sometimes can be a little laborious.

DR. SALTZMAN: You use a thermostated water bath.

MR. TOLIVER: And you can maintain your temperature to plus or minus 0.1 rather easily?

DR. SALTZMAN: No problem, the commercial equipment is available to a hundredth of a degree.

MR. WANDS: I would like to ask one question, Dr. Saltzman, what is the limiting factor in the age of these? Complete loss of the liquid contained in the tube or deterioration of the tube?

DR. SALTZMAN: Apparently it is the depletion of the liquid. Whether the tube is half full or completely full or almost empty, the permeation rate remains the same, it's the vapor pressure. You have a constant vapor pressure, you have to have liquid on the inside when the tube is completely depleted, you just throw it out.

MR. WANDS: Well, Teflon is known for its propensity to undergo cold flow, and I would think that perhaps the internal pressure might be enough to expand the tube at least slowly and thereby change your surface area and your wall thickness and thus the diffusion rate.

DR. SALTZMAN: Well, there are two types of Teflon that are used for the permeation tubes. TFE which is tetrafluoroethylene which is very porous and FEP which is fluorinated ethylene propylene, and gives perhaps a tenth of the permeation rate of TFE. Now I showed you the calibration data in which it appears that three weeks are required before a steady state is reached. And there is an equilibration period and perhaps it is due to mechanical flow. I might mention also that it seems as if you should anneal the Teflon, perhaps in the manufacturing process you get a highly unrepeatable condition of the Teflon, and if you hold it at 30° or even higher without blowing up the tube you can reach a steady state more rapidly.

DR. THOMAS: I was just thinking about your remark that this could be used also for exposure purposes. We've been talking about multiple, combined exposures to a number of contaminants. I realize that this would probably be too slow for a dome or something like that. But using small animals, what is your feeling of the kind of concentrations you could achieve with hydrocarbons for example with a reasonable flow rate in a small exposure chamber, so that you don't build up CO<sub>2</sub>, and other wastes that you really didn't want in there?

DR. SALTZMAN: Well, for example, in the case of nitrogen dioxide, which is a relatively fast permeating material, we're talking about quarter-inch tubes now, permeation rate of say 5 or 6 inch tube might be on the order of 10 say 20 microliters a minute, which means if you had a flow of one liter a minute, you have 30 parts per million in that tube. This gives you the ball park. Now, permeation rate is proportional to the exposed surface area of the Teflon and inversely proportional to the thickness and there is no reason why you couldn't design a permeation tube which would give you sufficient area to get any rate you wish.

DR. THOMAS: Can this be scaled up?

DR. SALTZMAN: No theoretical reason why it should not. You would have to provide sufficient mechanical strength to contain the pressure, sufficient area to get the rate you wish and, if necessary, a sufficient reservoir, a tank, glass, or stainless steel tank, to give you the lifetime you wish.

MR. WANDS: I think as a matter of fact, Anton, I have seen descriptions of large scale operations using finer bore Teflon tubes for water purification, brackish water purification through diffusion using the Teflon tube.

DR. SALTZMAN: I might mention that helium is commercially purified by permeation through glass tubes, so if you look at the commercial versions, I am sure that you could adapt them.

MR. WANDS: Are there any other questions either for Dr. Saltzman or any of the rest of our afternoon panel?

MR. VERNOT: I would like to ask Dr. Leon a question. It appeared to me that in his transformation of the data that he was getting from his fragiligraph there seemed to be transformation of transmission data from the optical portion into absorbance data. That's a log transformation. Then the dilution curve that he showed seemed to be an exponential dilution curve of the salts in the cuvette and this too could be linearized by a log transformation, and I wonder whether it could be accomplished, this transformation to linear data, simply by using a log-log, x-y recorder or something of this sort.

DR. LEON: I think that's what Mr. Toliver asked. I suppose it could be. Well, in the first place, the salt curve is not always the same, so you would have to have a slightly different equation to handle that part of it. The fact that it isn't always the same, and secondly because of the varying hematocrit--that is you don't always have the same hematocrit, the transmission, the percent hemolysis scale is not always the same. It is always a little bit different, that's the problem. If it were always the same, then it would be a simple process, like you say. I would just like to mention, for those of you who don't have access to computers, and we've thought of doing this, you could linearize this thing, just with a ruler if you want. Of course it will take you all day to get one through, but you could do it. If you have a graduate student or other form of slave labor, I suppose you could give it to them.

DR. BEARD: A question for Dr. Thomas--the technique which you described is a fascinating one, it starts from a very simple kind of observation, but puts it in terms that can be measured, and I think this is great. Can you do this without going through the step of time-lapse photography, I should think so?

DR. THOMAS: Yes, you can use ambient light for closed circuit TV pickup and put your sensors on the TV screen, and so the sampling rate can be increased tremendously. What is the scanning rate on TV, Barry?

MR. MYERS (Technology Incorporated): Actually, we synchronize off the horizontal synchronization rate of the TV itself, and I have forgotten the specific number, something like 16 milliseconds, I think.

DR. THOMAS: Another thing which you can do is to put on a timer and let the machine print out every hour for you.

DR. BEARD: Would it not be feasible, particularly if you were using smaller animals, to use some kind of a mask with sectors in it, not just two sectors, but multiple sectors and as animals move from the masked area into an open area they would then give a signal?

DR. THOMAS: You see, if you can sample with the TV synchronization rate, this is going to save a lot of eyeballs in drug studies, because if you sample that frequently in such rapid succession, you should be able to pick up convulsions, they would look like popcorn going off all over the place.

FROM THE FLOOR: Dr. Thomas, in your discussion, you made a comment that struck me as though you must have an error here. As the lighter colored bellies of the dogs are exposed, you are going to get a lesser density in your optical picture, and even though that dog might have moved, it would look as though there was a change, but how does this change differ from the contrast with the base floor of the cage?

DR. THOMAS: The reference area is a neutral gray, which is in the middle of the gradation scale. And so anything which changes the optical information in the activity area is due to something happening there. In other words if you kill five dogs and put them down there, there is not going to be a change ever. Since you always compare to the previous frame, it's fool-proof.

MR. TOLIVER: What would be the net effect in your study with small mice, shall we say, if you painted one side of the mice black, or in some way darkened one side of the mice and lightened the other side? Obviously you would get an accentuation of your data, would you not?

DR. THOMAS: The ideal thing is if you are going to drug studies, use white mice or rats in a black box. Contrast of course increases the sensitivity. Now, each group of Beagles we get is obviously different, so we have to get baselines on these animals in the same area where they are going to be used in the dome for a couple of days, before we start the exposure. Now, you still can compare them to other dogs, a control group, which has its own peculiarity of colors. But again, on the control group you're running each day's activity recording against their own baseline, so what I'm trying to say, is that I might have a perfectly normal control group showing, let's say, a total activity of 1500 per day continuous activity from time-lapse, and I might have an exposed group still normal on which the baseline is only 600, because they're less spotted or less colorful. The thing is that you have to have the baseline on the same animals before you expose them. What you are interested in is the cancellation of spurious decreases in spontaneous activity due to aging, obesity, etc. not related to toxicity.

MR. TOLIVER: O.K. The second thing, what would be the advantage of being able to monitor various differences in wave length change within the chambers for your activities? I'm suggesting that not only can you get information from telling the activity, you can also get perhaps more information by being able to tell what particular portion of the spectrum, the light spectrum as a function of heat and therefore activity one gets.

DR. THOMAS: Well, I figure you can use color filters.

MR. TOLIVER: I'm suggesting liquid crystals, obviously.

SESSION III

PATHOLOGY

Chairman

Dr. Frank M. Townsend  
University of Texas Medical School  
San Antonio, Texas

## MORPHOLOGY OF THE NON-DISEASED KIDNEY OF THE RHESUS MONKEY (MACACA MULATTA) WITH COMMENTS ON RENAL PHYSIOLOGY

C. Craig Tisher, M.D.

Duke University Medical Center  
Durham, North Carolina

### INTRODUCTION

The increased use of subhuman primates in biological research has made it mandatory for researchers to learn more about the morphology and physiology of these animals in non-diseased or steady states. The rhesus monkey (*Macaca mulatta*) represents a subhuman primate in which a wealth of such baseline biological information now available. The following paper addresses itself to the morphology of the kidney of this subhuman primate and, in addition, outlines certain parameters of renal function, particularly as they relate to the structure of the kidney. The discussion will include gross morphology as well as light and electron microscopic structural characteristics of the kidney. The material from which these observations were drawn consisted of young adult male and female rhesus monkeys weighing from 2.2 to 6.0 kg. A major goal of the light and electron microscopic studies was to determine the morphological alterations that result from the methods of procurement and preparation of percutaneous renal biopsies. Thus, in all animals used for ultrastructural studies, the renal tissue was prepared for electron microscopy by immersion fixation of percutaneous biopsies and by *in vivo* intravascular perfusion of the remaining intact kidney using several fixative and buffer combinations. Many of these observations are the subject of several recent publications (Tisher et al, 1969; Tisher and Rosen, 1967; Rosen and Tisher, 1968; Tisher et al, 1968; Tisher et al, 1970; Tisher et al, 1968; Tisher, *in press*) where the methods used in determining the status of renal function in individual animals and the method of tissue preservation employed for light and electron microscopic observations are described in detail.

### RESULTS

The kidney of a young adult rhesus monkey weighing 3.0 kg weighs approximately 6.5 grams. The ratio of the body weight to the kidney weight in young adult animals is approximately 400:1. As shown in figure 1, the rhesus monkey kidney is unipapillary in structure. It is quite evident on examination of the gross specimen that the renal medulla of this animal is poorly developed, largely due to the virtual absence of an inner medullary zone. Figure 2 is a light micrograph detailing the appear-

ance of a sagittal section of kidney in which the plane of section passed through the mid-pelvic surface exactly parallel to and centered in the long axis of the papilla. The renal cortex is easily identified and its lower border, the juxtamedullary boundary, is indicated by a single arrow. The remainder of the sagittal section below the arrow represents medulla. In a recent study relating the morphology of the kidney to its concentrating ability (Tisher, *in press*) it was found that in 21 kidneys obtained from 13 animals, 56% of the entire thickness of the sagittal section represented medulla. However, the inner medullary zone accounted for only 14% of the entire sagittal surface. Thus, the very abbreviated renal medulla of this animal is largely due to the absence of a well-developed inner medullary zone. Urinary concentration studies performed in our laboratory reveal that after 24 to 45 hours of complete fluid deprivation these animals can maximally concentrate their urine to  $1412 \pm 151$  mOsm/kg H<sub>2</sub>O (range 1126 to 1670 mOsm/kg H<sub>2</sub>O) (Tisher, *in press*). This compares quite favorably to man who can concentrate maximally to  $1000 \pm 200$  mOsm/kg H<sub>2</sub>O and possesses a kidney with a well developed inner medulla with long loops of Henle. This finding is of physiological significance since it has long been accepted that in mammals the ability to concentrate urine maximally is directly related to the length of the renal medulla and to the long loops of Henle (Berliner and Bennett, 1967). In short, the rhesus monkey represents a striking exception to this generally accepted rule. A more detailed discussion of this problem can be found in articles by Tisher et al, 1968; Tisher et al, 1970; and Tisher, *in press*.

Before beginning a presentation of the morphology of the individual components of the nephron, it should be noted that the term renal corpuscle rather than the term glomerulus will be used to designate that portion of the nephron which includes: 1) the capillary network lined by endothelial cells; 2) a central region of mesangial cells with continuous mesangial matrix material; and 3) visceral and parietal epithelial cells and their associated basement membranes. The basic structure of the renal corpuscle of the rhesus monkey kidney is similar to that of other laboratory animals and man. It is composed of the four basic cell types which include the endothelial cells that line the glomerular capillaries, the central stalk or mesangial cells, the visceral epithelial cells with their foot processes that come into contact with the external surface of the basement membrane of the peripheral capillary loops and the parietal epithelial cells that line Bowman's capsule. Figure 3 is a light micrograph denoting the relative position of these four cell types in the renal corpuscle of the rhesus monkey kidney, and figure 4 is an electron micrograph demonstrating the same features at higher resolution.



Figure 1. GROSS APPEARANCE OF THE CUT SURFACE OF A BIVALVED KIDNEY FROM A 3.4 KG RHESUS MONKEY. The boundary between the cortex and medulla is easily discernible. The papilla is blunted and opens into a simple type renal pelvis.



Figure 2. LOW POWER LIGHT MICROGRAPH SHOWING THE SAGITTAL SURFACE OF A BIVALVED RHESUS MONKEY KIDNEY. A single arrow denotes the cortico-medullary boundary at the level of the arcuate vessels. Gomori trichrome stain. X 4.2

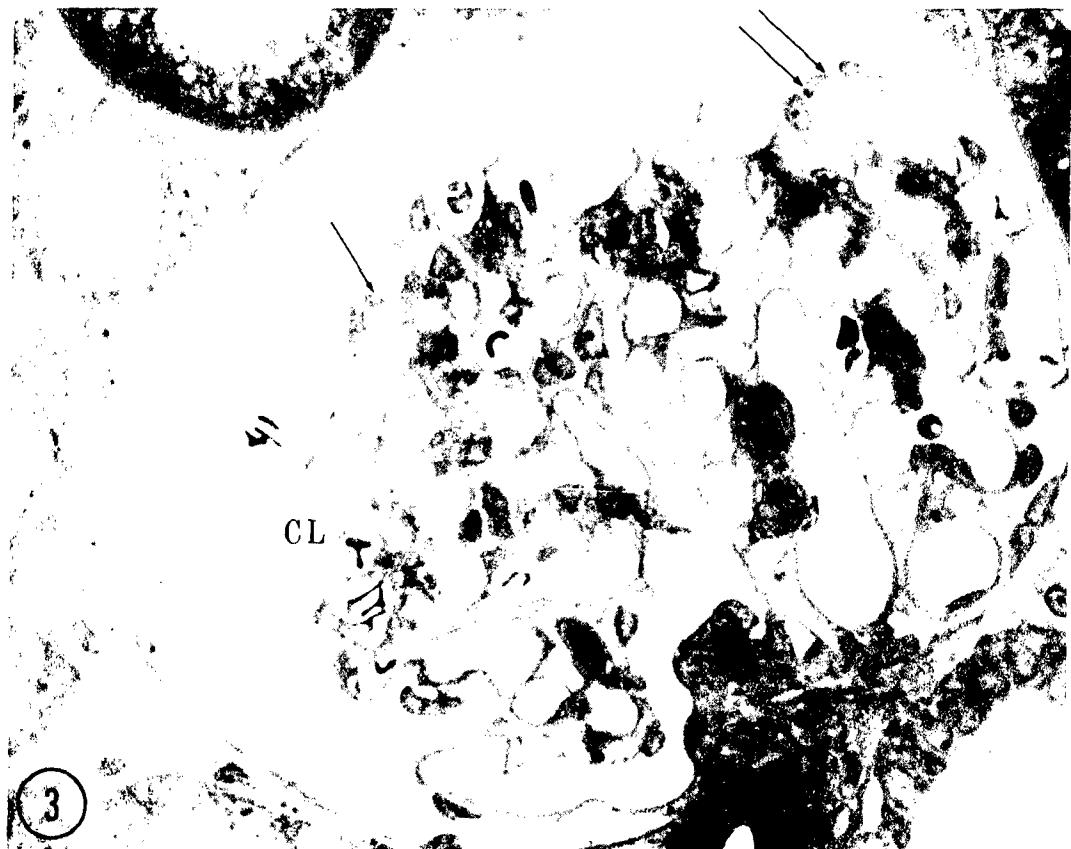


Figure 3. PHOTOMICROGRAPH OF A RENAL CORPUSCLE FROM A NON-DISEASED RHESUS MONKEY. The capillary lumens (CL) are patent and contain red blood cells. The mesangial cells (Me) are dark staining while the endothelial cells (arrow) and visceral epithelial cells (double arrow) are more pale staining. X 730



Figure 4. ELECTRON MICROGRAPH SHOWING PART OF A GLOMERULAR TUFT WITH CHARACTERISTIC MESANGIAL CELLS (Me), VISERAL EPITHELIAL CELLS (VE), AND ENDOTHELIAL CELLS (En). CL, capillary lumen. X 4,700

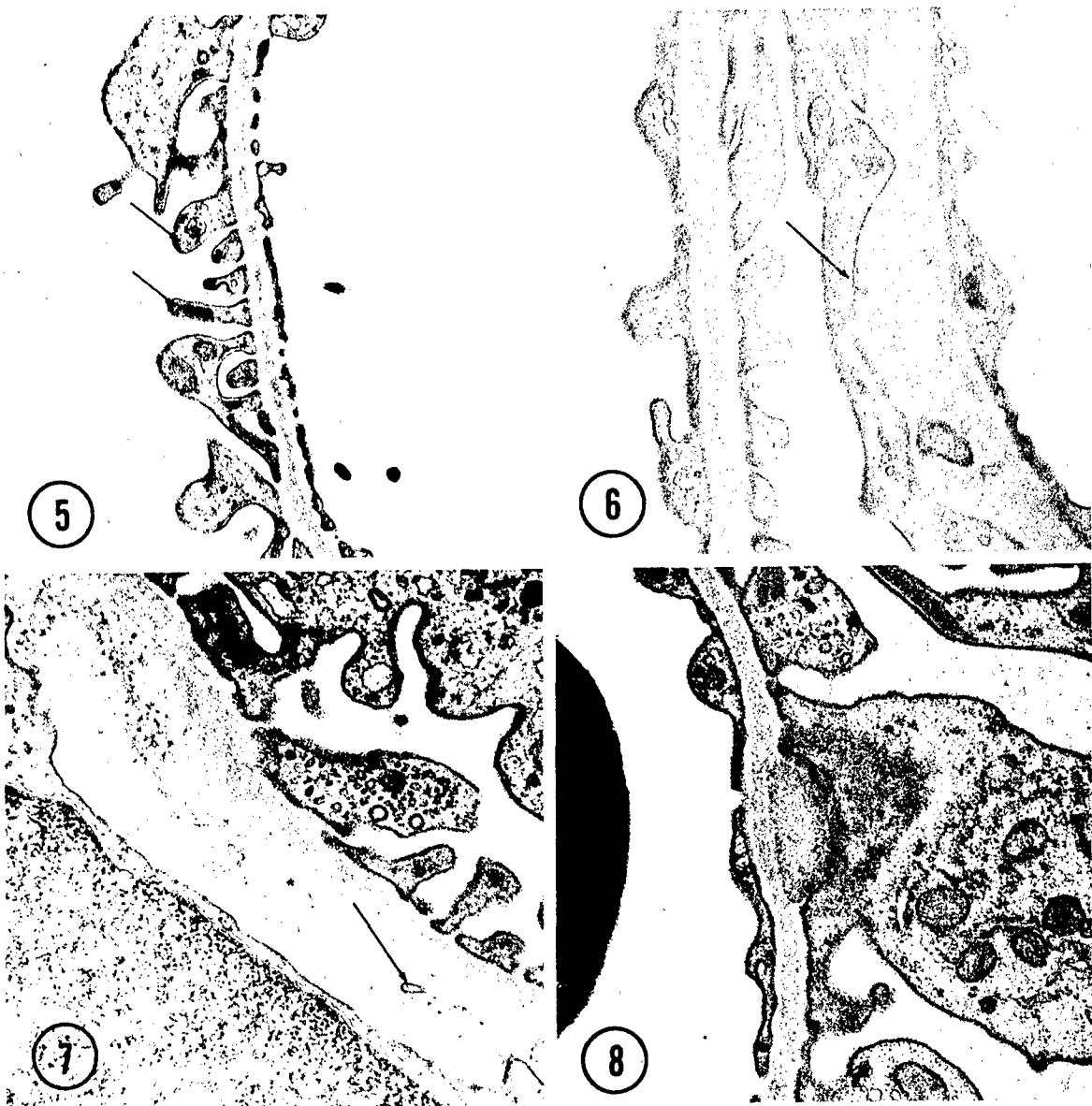


Figure 5. ELECTRON MICROGRAPH OF A SEGMENT OF NORMAL BASEMENT MEMBRANE. The foot processes (arrows) of the visceral epithelial cells are shown on the left and the attenuated endothelial cytoplasm is present to the right of the basement membrane. From left to right the three layers of the basement membrane include a lamina rara externa, a lamina densa, and a lamina rara interna. X 11, 350

Figure 7. ELECTRON MICROGRAPH SHOWING A CIRCULAR INCLUSION (ARROW) AND MEMBRANOUS AND FIBRILLAR FRAGMENTS WITHIN THE LAMINA DENSA OF THE PERIPHERAL BASEMENT MEMBRANE OF A RENAL CORPUSCLE. X 22, 750

Figure 6. ELECTRON MICROGRAPH SHOWING FOCAL THICKENING OF THE BASEMENT MEMBRANE OF A PERIPHERAL CAPILLARY LOOP OF A RENAL CORPUSCLE WITH ASSOCIATED FOOT PROCESS ALTERATIONS (ARROW). X 23, 100

Figure 8. ELECTRON MICROGRAPH OF A PORTION OF A PERIPHERAL CAPILLARY LOOP SHOWING A TYPICAL ELECTRON DENSE SUBEPITHELIAL HUMP. X 15, 400

Because the basic ultrastructure of the renal corpuscle of the rhesus monkey kidney is similar to that of mouse, rat and man (Rosen and Tisher, 1968) a detailed description will not be offered here. A few features of significance will be discussed, however. As in other animals the basement membrane of the peripheral capillary loops is composed of a central lamina densa and two peripheral electron lucent layers, a lamina rara externa and interna (figure 5). Each of the latter two layers comprises about one tenth of the total membrane thickness. The basement membrane has been found to be fairly uniform in thickness measuring approximately  $1840 \pm 290$  angstroms (standard deviation,  $N = 150$ ) (Rosen and Tisher, 1968). This thickness is considerably less than that of the human which has been found to be approximately 3288 angstroms (Jorgensen and Bentzon, 1968). Focal thickening of the basement membrane is occasionally observed. This is sometimes associated with localized alterations of the foot processes overlying the involved area (figure 6). Not infrequently small dense deposits and circular inclusions are observed in the lamina densa and occasionally in the lamina rara externa (figure 7). These usually measure 500-1800 angstroms in diameter, are at times membrane-limited, and are also related to focal foot process alterations. Infrequently, large hump-shaped subepithelial densities are observed, not unlike those reported in the renal corpuscles of patients with poststreptococcal glomerulonephritis (Strunk et al, 1964) or those induced by chronic injections of foreign protein (figure 8). It is possible that such deposits could reflect the handling of foreign protein that has entered the circulation such as that associated with extensive testing for tuberculosis, a common procedure these animals have undergone. Whatever the etiology of these densities, their presence in nondiseased animals serves to illustrate the difficulty and pitfalls that occur in identification of glomerular disease processes with the aid of the electron microscope.

Certain variations are observed within the cellular components of the rhesus monkey renal corpuscle which deserve additional emphasis. Crystalline structures measuring 0.2 to 0.7 microns are found within endothelial cells (Rosen and Tisher, 1968; De Martino et al, 1969). They are composed of a lattice network formed by 250 to 300 angstrom circular or possibly hexagonal elements that interconnect with each other (figure 9). Similar structures are also present in the endothelial cells of peritubular capillaries (figure 10). The inclusions are usually in intimate contact with the endoplasmic reticulum. They have also been described in the endothelium of pulmonary and hepatic capillaries of rhesus monkeys (De Martino et al, 1969). Similar structures have been reported in the endothelium lining capillaries within the lamina propria of the ileum and transverse colon of the cynos monkey (*Macaca irus*) (Kanamitsu et al, 1967) and within monkey kidney cell cultures infected with rubella virus (Kim and Boatman, 1967). It is not clear what the significance of these endothelial crystalline inclusions is. It does not seem likely that this is a secretion product. The possibility that this structure represents some type of viral inclusion within the endothelial cell cannot be excluded at this time.

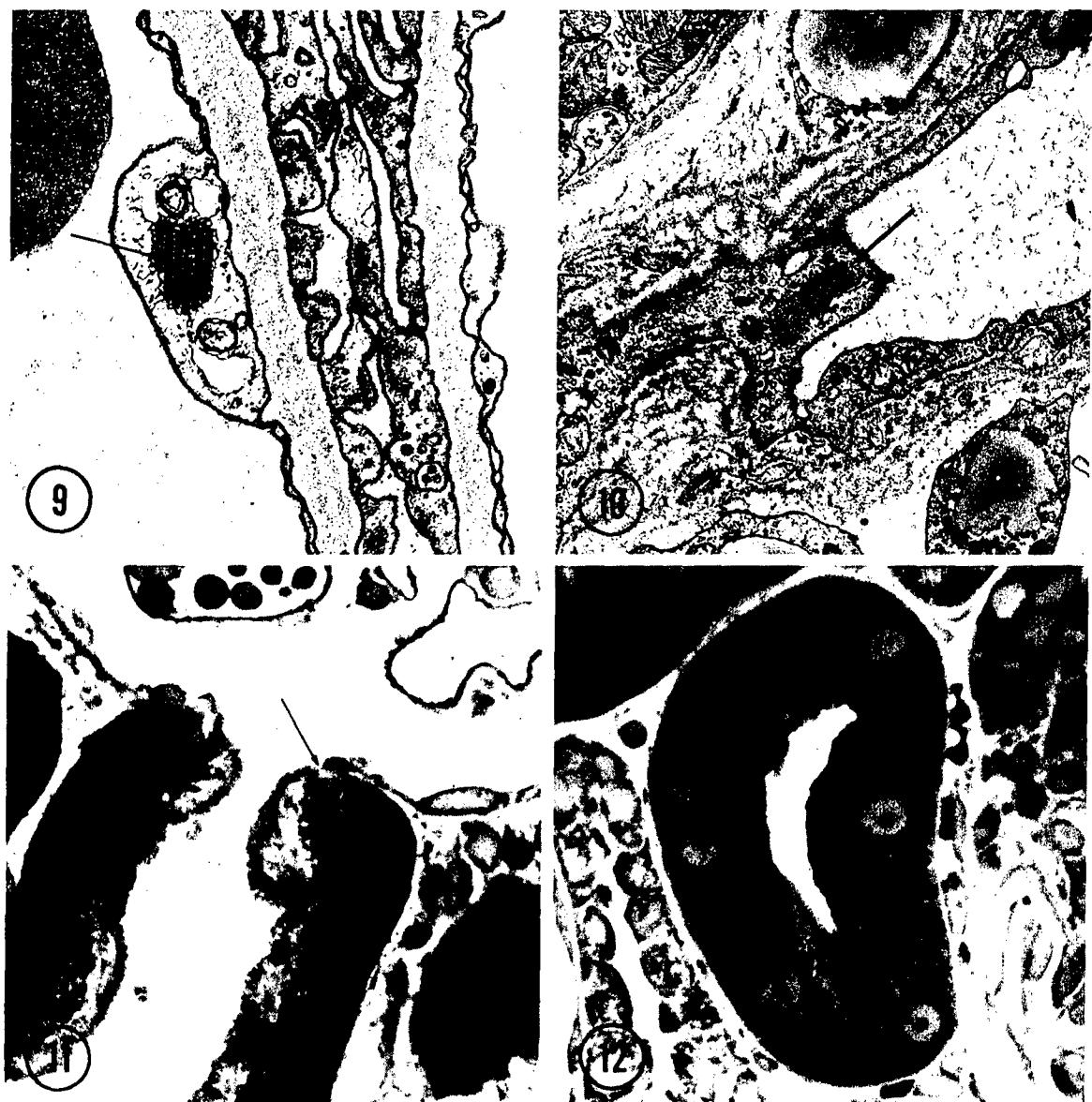


Figure 9. ELECTRON MICROGRAPH DEMONSTRATING A CRYSTALLINE INCLUSION (ARROW) WITHIN A GLOMERULAR ENDOTHELIAL CELL. X 21,600

Figure 10. CRYSTALLINE INCLUSION (ARROW) IN AN ENDOTHELIAL CELL OF A PERITUBULAR CAPILLARY WHICH IS IDENTICAL IN APPEARANCE TO THAT WITHIN THE ENDOTHELIUM LINING GLOMERULAR CAPILLARIES. (See figure 9 for comparison). X 12,300

Figure 11. PHOTOMICROGRAPH DEPICTING THE ABRUPT TRANSITION (ARROW) FROM LOW LYING SQUAMOUS EPITHELIUM LINING BOWMAN'S CAPSULE TO TALL COLUMNAR EPITHELIUM CHARACTERISTIC OF THE FIRST SEGMENT OF THE PROXIMAL TUBULE. X 500

Figure 12. PHOTOMICROGRAPH OF THE FIRST SEGMENT OF THE PROXIMAL TUBULE. The epithelium is tall and columnar, the brush border is well developed, and the cells are packed with elongate mitochondria. X 700

In the proximal tubule of the rhesus monkey, three distinct segments are identifiable by both light and electron microscopy. The segments can be defined by the morphology of their individual cellular components and by their relationship to other regions of the nephron. The first segment, which in general corresponds to the pars convoluta of the proximal tubule begins as an abrupt transition from the flattened squamous epithelial cells lining Bowman's capsule (figure 11). The cells are tall columnar and exhibit a well-developed PAS positive brush border and numerous elongate mitochondrial profiles (figure 12). Cells of the middle or second segment of the proximal tubule are low columnar in shape and possess a shorter and often more irregular brush border than those of the first segment (figure 13). Mitochondria are generally more tortuous and seldom elongate. Lipid droplets are most commonly observed in the second segment and situated along the base of the cell. The third segment of the proximal tubule corresponds in general to the pars recta or pars descendens. The cells of this segment are cuboidal in shape, exhibit a convex luminal surface and are covered by a distinct brush border. These cells contain far fewer organelles than cells of the first two segments of the proximal tubule and are generally less complex in structure (figure 14). The transition from the terminal proximal tubule to the thin descending limb of Henle is generally gradual and marked by the accumulation of large deposits of lipofuscin or degeneration pigment (figure 15).

Figures 16 through 19 represent electron micrographs of the neck region and the three segments of the proximal tubule. Since the fine structure of these cells does not vary greatly from that observed in the human kidney and in other laboratory animals, a detailed discussion will not be presented here. See reference 21 for further descriptions. The general cell shape, size, and configuration of the proximal tubule cells is nearly identical in man and rhesus monkey. One difference, however, is the presence of occasional distinct outpouching or evaginations at the base of proximal tubule cells in the monkey (figure 20). These structures are present in the first two segments of the tubule and are often associated with bands of coarse fibrils which extend across the mouth or neck of these evaginations. Similar structures have been observed in the proximal tubule of the rat (Strunk, 1964). Their structural and functional significance, if any, is unknown.

The microbody is the most common single membrane-limited inclusion body (SMLIB) in the rhesus monkey proximal tubule (Tisher et al, 1969), whereas cytosomes are the most common SMLIB present within the proximal tubule of the human kidney (Svoboda and Higginson, 1964). The structure of the microbody in the monkey is considerably different from that of man. In addition, a considerable variation in the appearance of microbodies in the monkey kidney is evident, depending upon the type of fixation that is employed. When tissue is preserved by in vivo intravascular perfusion of fixative, the microbodies are elongate in structure and possess one or more marginal plates along their outer limiting membrane (figure 21). When the tissue is preserved by immersion fixation, the marginal plates are often broken or fractured resulting in grotesquely angulated structural configurations (figures 22 and 23). See references 20 and 21 for a more detailed discussion. The renal microbodies in the human are usually spherical in structure with less prominent marginal plates (Tisher et al, 1966). It should be emphasized that thus far microbodies have only been found in the proximal tubule of the mammalian nephron.

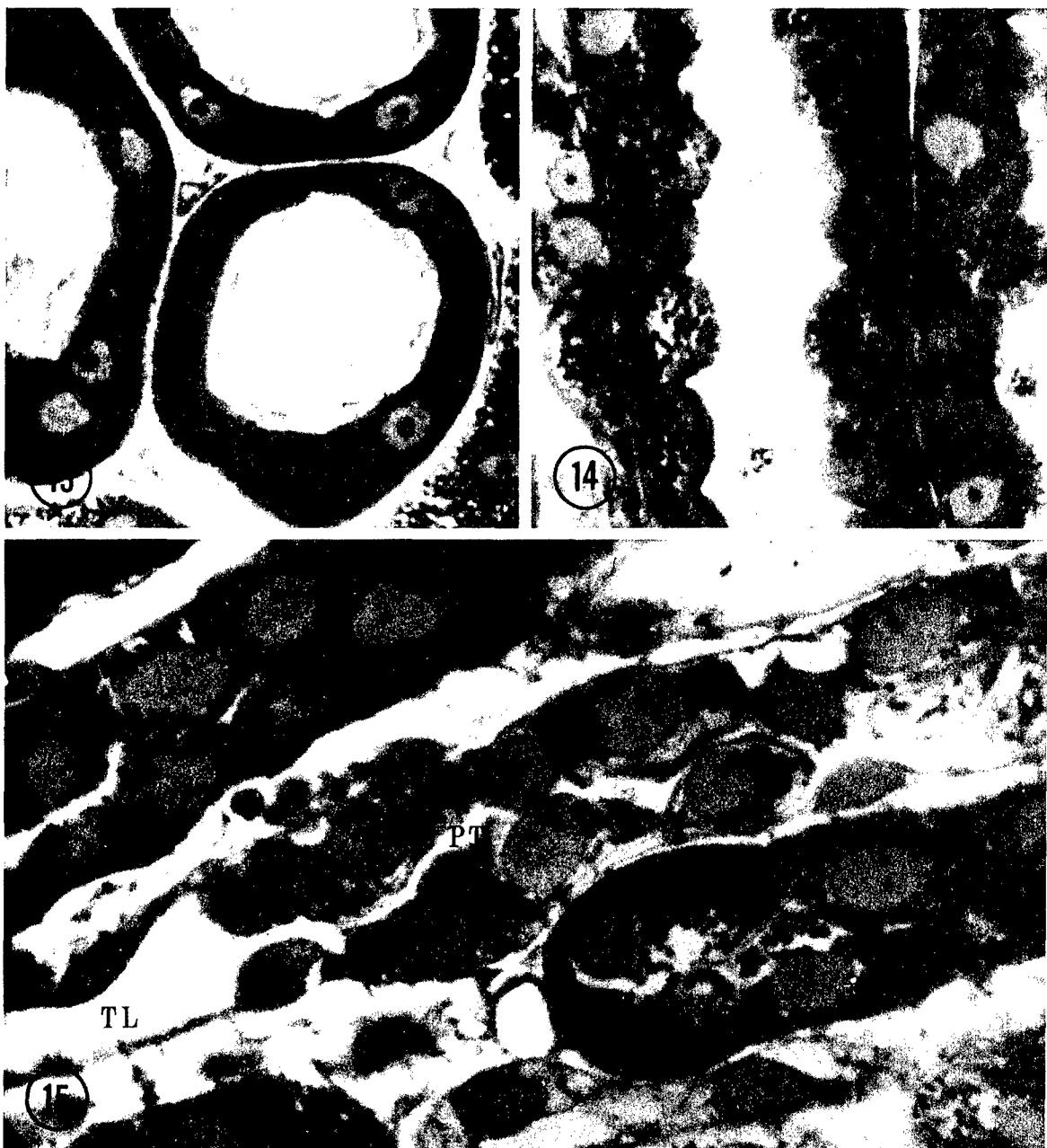


Figure 13. PHOTOMICROGRAPH OF THREE ADJACENT TUBULES CHARACTERISTIC OF THE SECOND SEGMENT OF THE PROXIMAL TUBULE. X 800

Figure 14. PHOTOMICROGRAPH CHARACTERISTIC OF THE THIRD SEGMENT OF THE PROXIMAL TUBULE. Cells are cuboidal in shape and have a convex luminal border. X 800

Figure 15. PHOTOMICROGRAPH SHOWING THE CHARACTERISTIC GRADUAL TRANSITION FROM THE TERMINAL PROXIMAL TUBULE (PT) ON THE RIGHT TO THE EARLY DESCENDING THIN LIMB (TL) OF HENLE'S LOOP ON THE LEFT. X 890



Figure 16. ELECTRON MICROGRAPH DEMONSTRATING TRANSITION FROM SQUAMOUS EPITHELIUM (ARROW) LINING BOWMAN'S CAPSULE TO THE COLUMNAR CELLS OF THE PROXIMAL TUBULE ABOVE. BS, Bowman's space; BM, basement membrane; RBC, red blood cell; VE, visceral epithelial cell. X 9250



Figure 17. ELECTRON MICROGRAPH OF A TALL COLUMNAR CELL FROM THE FIRST SEGMENT OF THE PROXIMAL TUBULE. Elongate mitochondrial profiles (M) are enclosed in plications of the basal plasmalemma. The apical system of vesicles, vacuoles and dense tubules is well-developed. PC, peritubular capillary; AV, apical vacuole. X 10,000

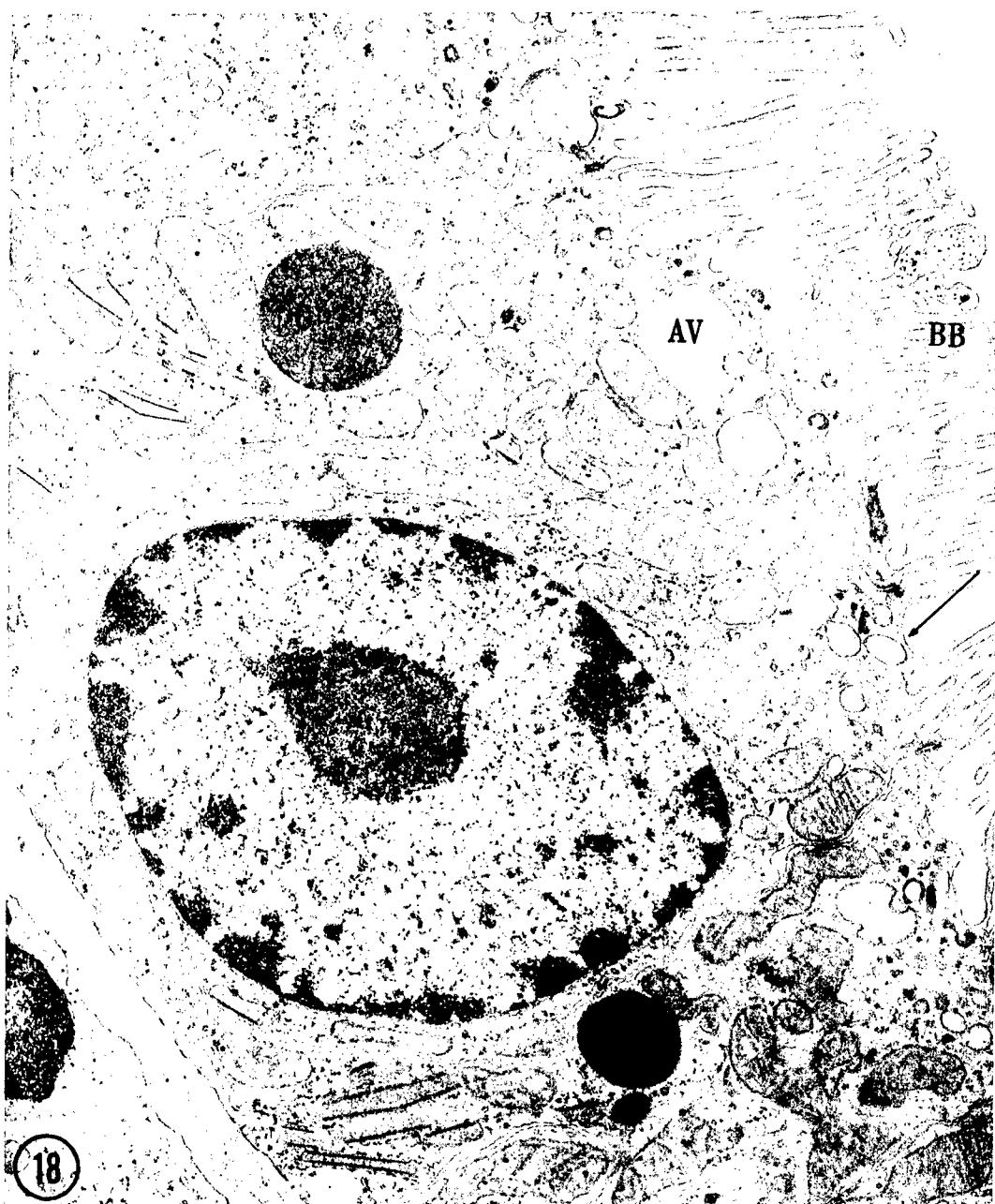
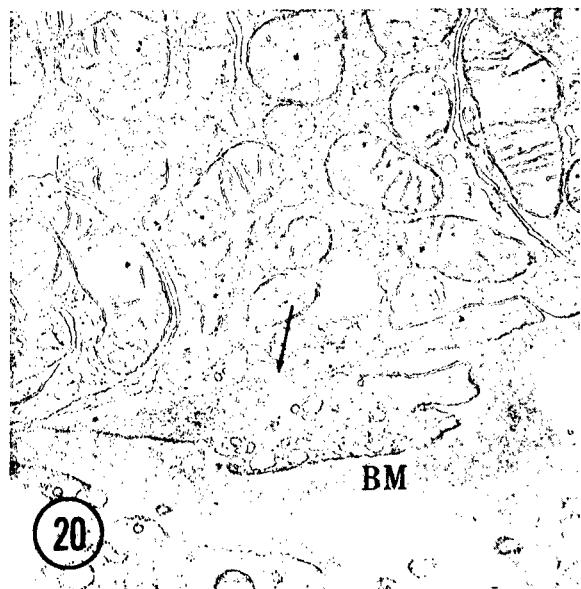


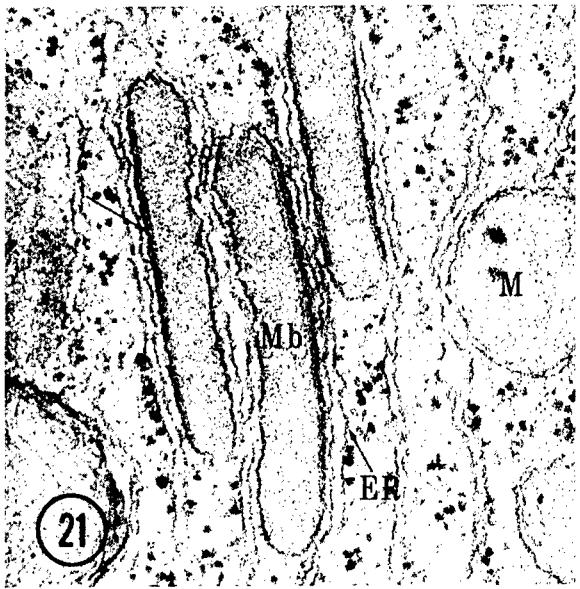
Figure 18. ELECTRON MICROGRAPH OF A PROXIMAL TUBULE CELL FROM THE SECOND SEGMENT. The brush border (BB) is more irregular than that of the first segment and occasional skip areas (arrow) are noted. Apical vesicles and dense tubules are not as extensively developed but apical vacuoles (AV) are often more prominent. The cell is low columnar and lateral interdigitations with adjacent cells are less complex when compared to the first segment. C, cytosome. X 13,175.



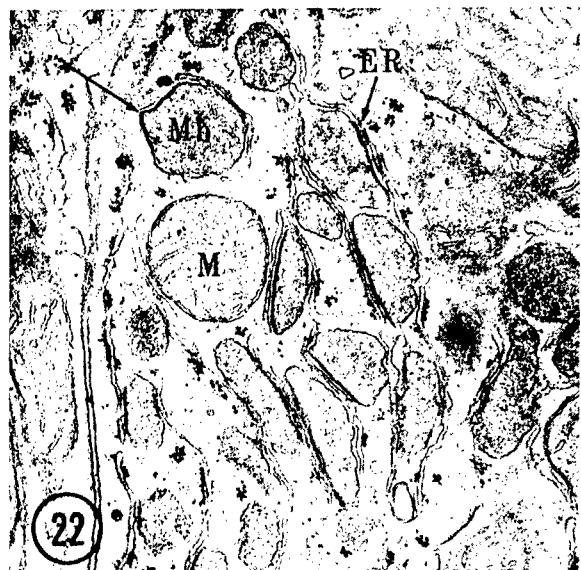
Figure 19. ELECTRON MICROGRAPH SHOWING A PROXIMAL TUBULE CELL CHARACTERISTIC OF THE THIRD SEGMENT. The cells are cuboidal and exhibit a well-developed brush border. Apical dense tubules and apical vacuoles are not extensive in this segment, although small apical vesicles are abundant. Microbodies (Mb) are the most common SMLIB in this segment. Note the relatively thin basement membrane (arrow). IC, interstitial cell. X 10,500



20



21



22

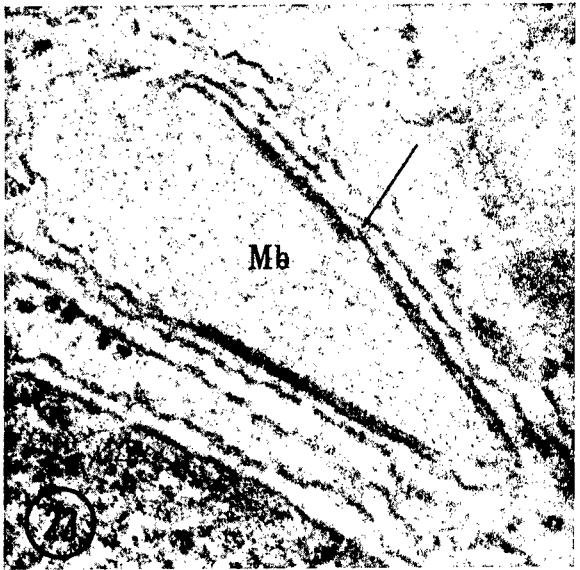


Figure 20. ELECTRON MICROGRAPH SHOWING AN EVAGINATION OR OUTPOUCHING AT THE BASE OF A PROXIMAL TUBULE CELL. Such structures are seen in both first and second segments of the proximal tubule in perfusion-fixed tissue. Bands of coarse fibrils (arrow) usually extend across the mouth of these evaginations running parallel to the adjacent basement membrane (BM). X 14,000

Figure 21. ELECTRON MICROGRAPH SHOWING A CLUSTER OF ELONGATE MICROBODIES (Mb) FROM A CELL OF THE FIRST SEGMENT OF THE PROXIMAL TUBULE. The tissue was preserved by in vivo intravascular perfusion of 1 percent osmium tetroxide. Each organelle possesses one or two dense marginal plates (arrow) contiguous with the adjacent endoplasmic reticulum (ER). M, mitochondrion. X 56,000

Figure 22. ELECTRON MICROGRAPH SHOWING A LARGE CLUSTER OF MICROBODIES IN KIDNEY TISSUE OBTAINED BY PERCUTANEOUS RENAL BIOPSY AND PRESERVED BY IMMERSION FIXATION IN 1 PERCENT OSMIUM TETROXIDE. The microbodies (Mb) maintain the same intimate relationship and orientation to the endoplasmic reticulum (ER) as shown in figure 21 but are extremely variable in size and shape and exhibit distortion and acute angulation of the marginal plates (arrow). M, mitochondrion. X 24,000

Figure 23. HIGH POWER ELECTRON MICROGRAPH DEMONSTRATING A FRACTURED MARGINAL PLATE (ARROW) OF A MICROBODY (Mb) WITH SUBSEQUENT DISPLACEMENT OF THE TWO ENDS. The tissue was preserved by immersion fixation. X 96,000

Two types of intramitochondrial inclusions are present in mitochondria of the rhesus monkey proximal tubule cells. The first type is composed of helical structures that lie entirely within widened cristae and are less electron dense than the surrounding mitochondrial matrix (figure 24). The inclusions are composed of filaments 30 to 40 angstroms in width. The diameter of the helix formed by the filaments is 130 to 140 angstroms with a pitch of approximately 120 angstroms. The coiled filaments usually run parallel to one another and often appear to twist or coil on each other. This was the most common type of mitochondrial inclusion observed in this material. Similar inclusions have been reported in mitochondria of astrocytes of the rat corpus striatum (Mugnaini, 1964) and within liver mitochondria following alcohol ingestion (Iseri et al, 1966; Porta et al, 1965). They have also been observed in hepatic cells of rats fed protein deficient diets (Svoboda and Higginson, 1964). The second type of inclusion lies free within the mitochondrial matrix and may represent a modification of the mitochondrial cristae (figure 25). It is composed of several parallel rows of membranes that resemble cristae. A regular periodicity of approximately 290 angstroms is present along these membranous profiles which appears to be the result of twisting of two of the parallel membranes to form a double helix. Additional details of this type of intramitochondrial inclusion can be found in reference 21. The significance of either of the two types of inclusions is not apparent but their presence in apparently normal non-diseased kidneys must be kept in mind when evaluating kidneys from experimental animals.

The thickness of the basement membrane of the proximal tubule in the rhesus monkey kidney decreases markedly from the first to the third segment. As shown on table I the average minimal thickness of the basement membrane of the first, second, and third segments is 2550 angstroms, 1460 angstroms and 703 angstroms, respectively. These findings emphasize the importance of identifying as precisely as possible the region of the proximal tubule under study in any investigation in which conclusions are being drawn regarding the significance of the thickness of the basement membrane and its possible relationship to a pathological condition. The thickness of the basement membrane also varies considerably depending upon the type of fixative and the method of fixation employed. As shown in table II, the basement membranes of proximal tubules fixed by immersion in osmium tetroxide have the greatest thickness, ranging from 1644 to 9799 angstroms. The mean values of the thinnest and thickest regions are  $3578 \pm 315$  angstroms and  $5692 \pm 340$  angstroms, respectively. In contrast, the mean values of the thinnest and thickest regions of the basement membrane of proximal tubules fixed by immersion in glutaraldehyde are  $2569 \pm 168$  angstroms and  $4280 \pm 263$  angstroms, respectively. As shown on table II, basement membrane thickness is nearly identical if proximal tubules are perfusion-fixed with osmium tetroxide or glutaraldehyde. Again, in any study in which conclusions are being drawn regarding the possible significance of the thickness of the basement membranes, the type of fixative employed and particularly the method of fixative application must be taken into consideration. A more detailed discussion of this problem can be found in reference 21.

TABLE I

## COMPARISON OF BASEMENT MEMBRANE THICKNESS IN THREE SEGMENTS OF THE PROXIMAL TUBULE OF THE RHESUS MONKEY

Segment	No. of Measurements	Minimum thickness		Maximum thickness	
		Mean $\pm$ SEM (Å)	Range (Å)	Mean $\pm$ SEM (Å)	Range (Å)
First	56	2550* $\pm$ 107	1257-4470	3845* $\pm$ 154	2184-6515
Second	56	1460* $\pm$ 83	680-3333	2672* $\pm$ 137	1162-5833
Third	56	703 $\pm$ 28	363-1101	1523 $\pm$ 67	731-3252

\*  $p = < 0.001$  compared with third segment.

TABLE II

## COMPARISON OF BASEMENT MEMBRANE THICKNESS OF THE FIRST SEGMENT OF THE PROXIMAL TUBULE FIXED IN GLUTARALDEHYDE AND OSMIUM TETROXIDE

Fixative	Method of Fixative Application	Minimum Thickness			Maximum Thickness		
		No. of Measurements	Mean, in Å ( $\pm$ S.E.M.)	Range, in Å	No. of Measurements	Mean, in Å ( $\pm$ S.E.M.)	Range, in Å
Glutaraldehyde	Immersion	28	2569 $\pm$ 168	1302-4039	28	4280 $\pm$ 263	2344-7374
Osmium Tetroxide	Immersion	28	3578* $\pm$ 315	1644-7308	28	5692† $\pm$ 340	2898-9799
Glutaraldehyde	IVIP‡	30	2518 $\pm$ 154	1257-4258	30	4013 $\pm$ 180	2204-6194
Osmium Tetroxide	IVIP‡	26	2588 $\pm$ 153	1502-4470	26	3650 $\pm$ 261	2184-6515

\*  $P = 0.01$

†  $P = 0.001$

‡ in vivo intravascular perfusion

As discussed in an earlier section of this paper, the inner medulla of the kidney of the rhesus monkey is poorly developed. As a result, long loops of Henle, defined as those loops entering the inner medulla before forming the "hairpin" turn, are virtually absent. Thus, a long ascending thin limb segment is unusual. The overwhelming majority of the loops of Henle are short and do not enter the inner medulla. There is usually a very short descending thin limb segment but the "hairpin" turn is most often formed by tubular epithelium characteristic of the ascending thick limb of Henle (figure 26). The fine structural characteristics of the thin descending limb (figure 27) and the thick limb of Henle (figure 28) are nearly identical to other mammals including man. The typical thin limb cell is squamous in appearance and usually exhibits numerous short blunt microvilli on the apical or luminal cell surface. The cell bulges into the lumen in the region of the nucleus, but otherwise the cell cytoplasm is quite attenuated. Complicated lateral interdigitations exist between adjacent cells. Near the junction with the terminal proximal tubule, thin limb cells often contain large collections of lipofuscin or degeneration pigment (figure 29). Similar appearing collections of pigment have been noted in the human thin limb (Bulger et al, 1967). Cellular organelles are relatively sparse in this segment but qualitatively are identical to other segments of the nephron distal to the proximal tubule.

The epithelium of the thick limb of Henle is cuboidal and contains abundant quantities of mitochondria. The mitochondria are elongate and usually enclosed by plications of the basal plasmalemma (figure 28). The invaginations of the basal plasmalemma extend two-thirds of the distance toward the apical cell surface. Interdigitations of lateral and basilar cell processes between adjacent cells are complex and extensive. A prominent feature of this segment of the nephron is the presence of numerous small coated and non-coated apical vesicles located just beneath the apical plasmalemma (figure 30). The structure of this segment, in particular, the numerous mitochondria intimately associated with the basal plasmalemma correlates well with the active sodium transport capability known to be a functional characteristic of this segment of the nephron (Berliner and Bennett, 1967; Bennett et al, 1968).

After the thick ascending limb segment of the loop of Henle enters the cortical region, the tubule comes into contact with the same renal corpuscle from which the proximal tubule of that nephron originated. At the point of contact a specialized region of the distal tubule, the macula densa, is formed (figure 31). The most outstanding feature of this region is the change in polarity of the individual cells. The nucleus is apically-placed and the arrangement of organelles such as the Golgi apparatus, cytosomes, multivesicular bodies and cytosegresomes appears to be indicative of secretory or resorptive activity directed away from the lumen and toward the cell base as originally suggested by Goormaghtigh (1939). The appearance of the macula densa in the rhesus monkey is similar to other laboratory animals and man and will not be discussed in further detail. The macula densa along with granular myoepithelioid cells located in the wall of the terminal afferent arteriole and the lacis cells situated between the macula densa and the renal corpuscle form the juxtaglomerular apparatus (figure 32). Large "secretory" granules that probably represent renin or renin precursors are located within the myoepithelioid cells of the afferent arteriole and cells with features resembling both the myoepithelioid and lacis cells (figure 33). Similar granules are

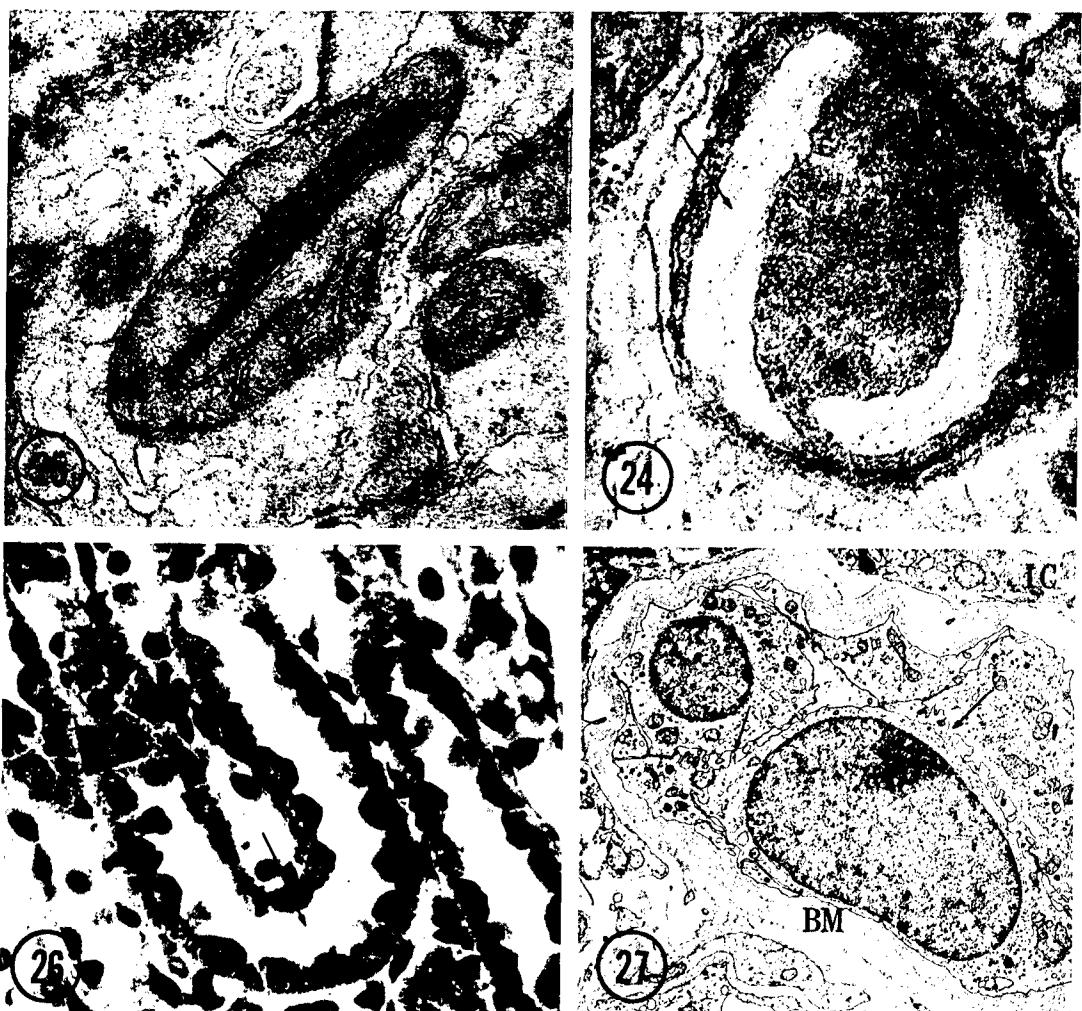


Figure 25. ELECTRON MICROGRAPH SHOWING A SECOND TYPE OF MITOCHONDRIAL INCLUSION (ARROW) WHICH IS MORE DENSE THAN SURROUNDING STRUCTURES AND COMPOSED OF SEVERAL PARALLEL ROWS OF MEMBRANES WHICH RESEMBLE MITOCHONDRIAL CRISTAE. See text for description. X 13,800

Figure 26. PHOTOMICROGRAPH FROM THE OUTER MEDULLA DEMONSTRATING A "HAIRPIN" TURN (ARROW) OF THE LOOP OF HENLE FORMED ENTIRELY BY EPITHELIUM CHARACTERISTIC OF THE THICK SEGMENT. Cells are cuboidal and contain large numbers of mitochondria and other cellular organelles. Gomori trichrome stain. X 640

Figure 24. ELECTRON MICROGRAPH SHOWING MITOCHONDRIAL INCLUSION (ARROW) LYING ENTIRELY WITHIN A WIDENED CRISTAE. See text for description. X 47,400

Figure 27. ELECTRON MICROGRAPH SHOWING THE APPEARANCE OF A THIN DESCENDING LIMB SEGMENT IN THE OUTER MEDULLA. Lining cells are simple squamous in type and contain few mitochondria or other organelles. Small blunt microvilli are numerous on the luminal cell surface (arrow). BM, basement membrane; IC, interstitial cell with lipid inclusion. X 4600

not observed within lacis cells, cells of the macula densa, or the efferent arteriole. Both circular homogenous electron-dense cytosomes and cytosomes containing oblong "granules" measuring up to 6 microns in length (figure 34) are seen. The oblong structures contain a central dense core with a highly ordered crystalline structure exhibiting a regular periodicity of approximately 80 angstroms. The central core is surrounded in most instances by a less dense homogeneous matrix.

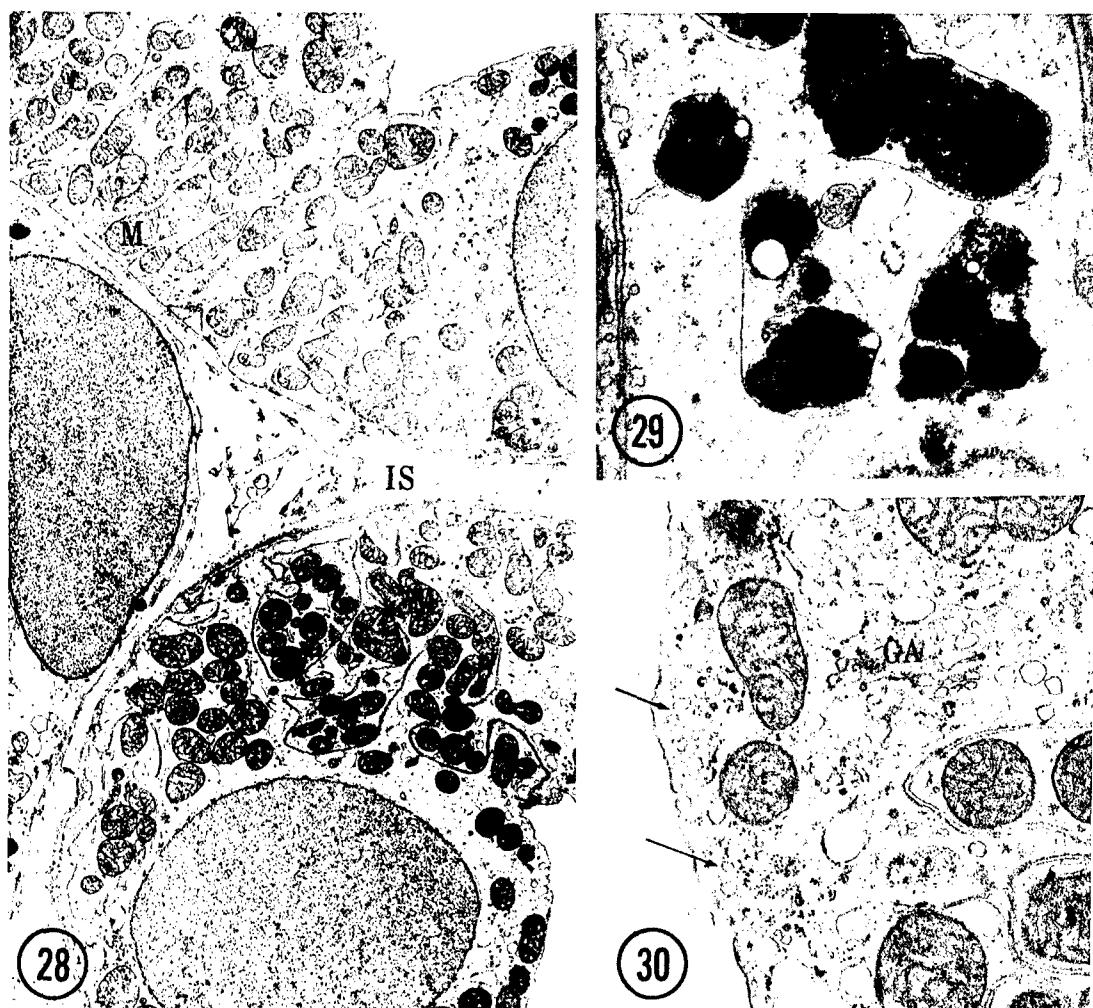


Figure 28. ELECTRON MICROGRAPH OF THICK LIMB SEGMENTS IN THE OUTER MEDULLA. The cells are cuboidal and contain numerous mitochondria (M), many of which are in intimate association with invaginations of the basal plasmalemma. IS, interstitial space. X 4500

Figure 29. ELECTRON MICROGRAPH SHOWING LARGE COLLECTIONS OF LIPOFUSCIN OR DEGENERATION PIGMENT IN THIN LIMB SEGMENTS OF THE RHESUS MONKEY KIDNEY. X 16,450

Figure 30. ELECTRON MICROGRAPH SHOWING THE NUMEROUS VESICLES (ARROWS) IN THE APICAL REGION OF A CELL TYPICAL OF THE ASCENDING THICK LIMB OF HENLE. GA, Golgi apparatus. X 12,800

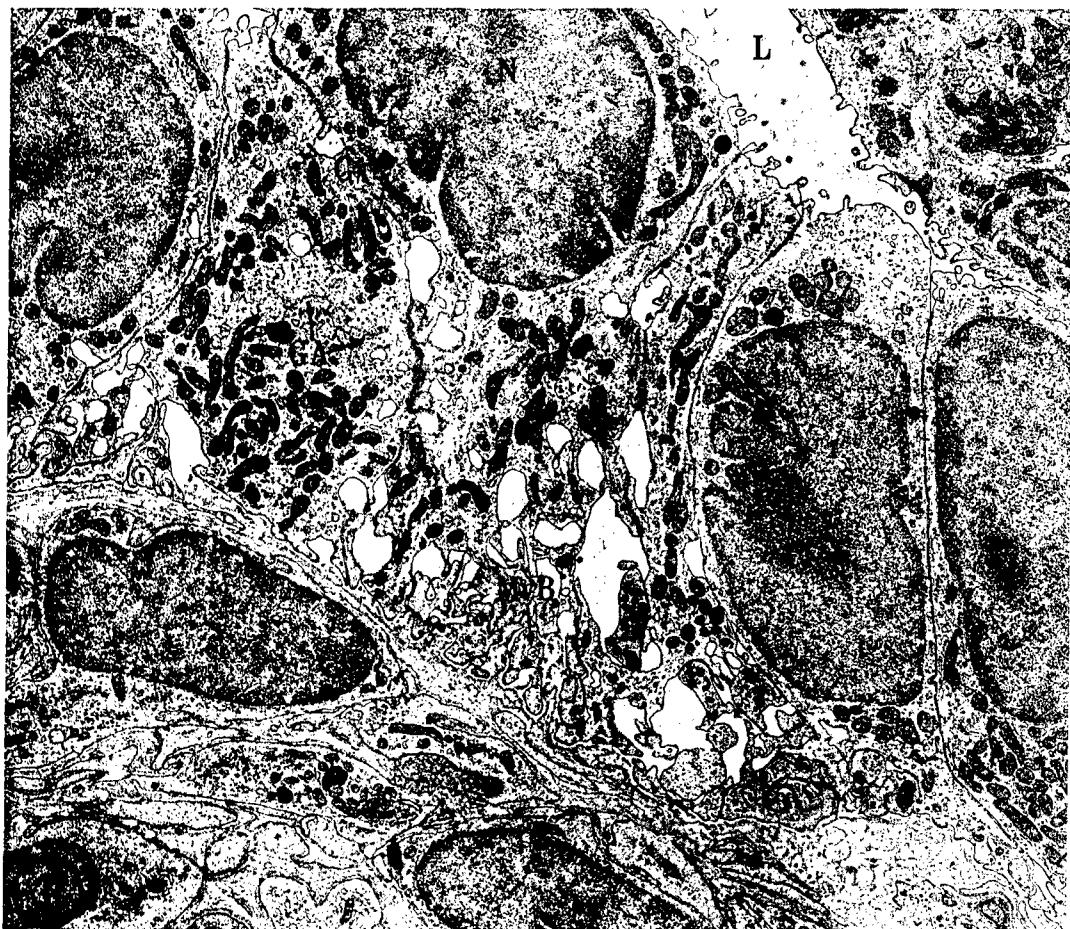


Figure 31. ELECTRON MICROGRAPH SHOWING THE STRUCTURE OF TYPICAL MACULA DENSA CELLS. The nuclei (N) are apically-placed and other cellular organelles including the Golgi apparatus (GA), cytosomes (C), and multivesicular bodies (MvB) are located toward the basal surface of the cell suggesting a reversed functional polarity. See text for possible functional significance. L, lumen of tubule. X 4,000

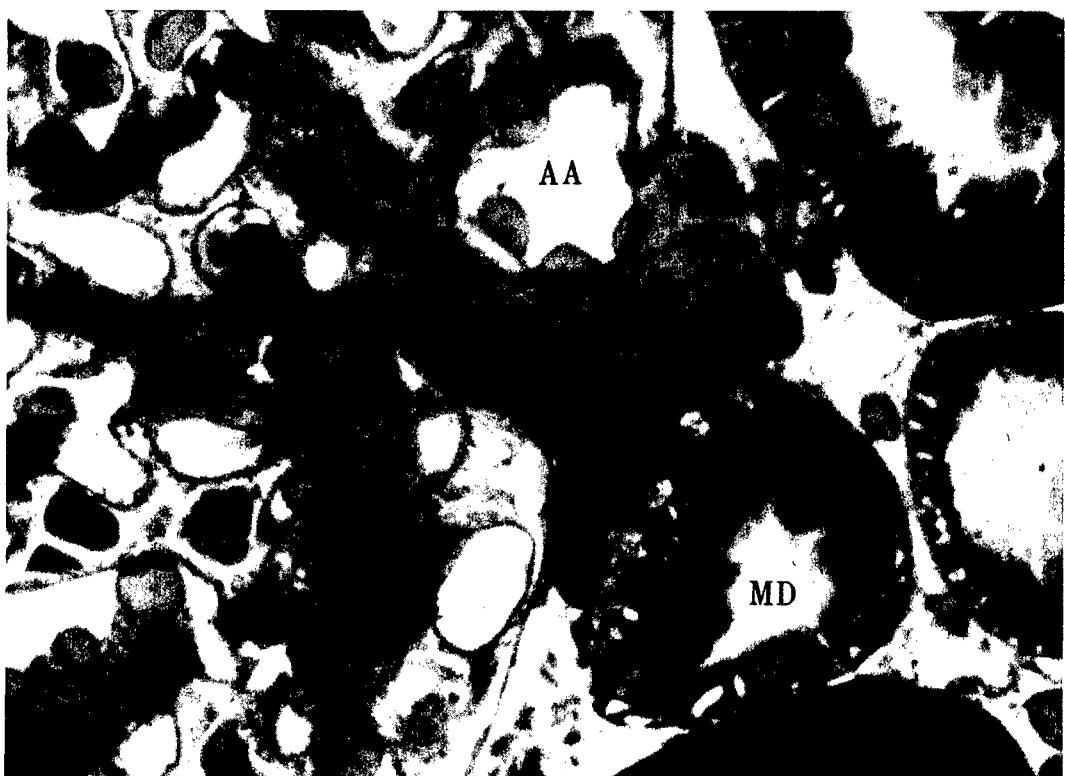


Figure 32. PHOTOMICROGRAPH SHOWING THE TYPICAL APPEARANCE OF A JUXTA-GLOMERULAR APPARATUS WHICH INCLUDES THE MACULA DENSA (MD), THE AFFERENT ARTERIOLE (AA) AND THE LACIS CELLS (LC) IN THE REGION OF THE POLKISSEN. X 525

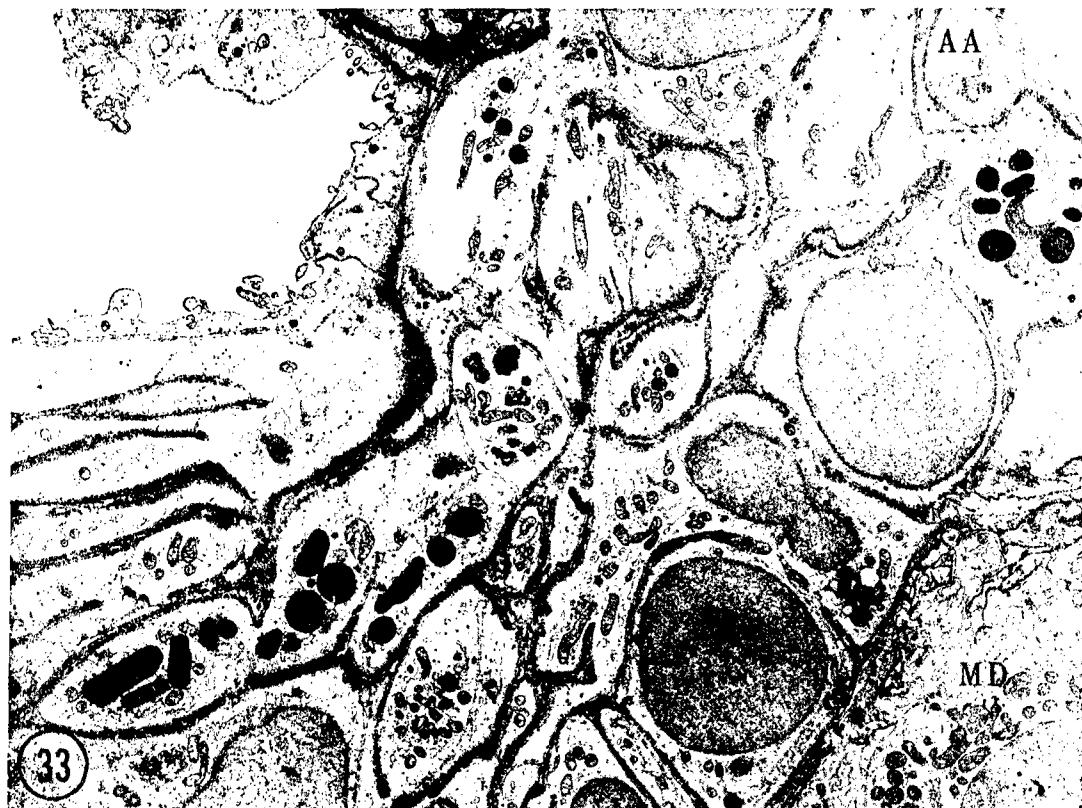


Figure 33. ELECTRON MICROGRAPH OF PORTIONS OF A JUXTAGLOMERULAR APPARATUS SHOWING THE AFFERENT ARTERIOLE (AA) AT THE UPPER RIGHT AND THE MACULA DENSA (MD) AT THE LOWER RIGHT. Numerous dense secretory granules are present within myoepithelioid and other contiguous cells. X 4,500

The distal convoluted tubule, extending from the macula densa to the connecting segment of the cortical collecting tubule, is similar in structure to the ascending thick limb of Henle. The cells are cuboidal to low columnar and easily identified by their large complement of elongate mitochondria enclosed within plications of the basal plasmalemma (figures 35 and 36). Dark or intercalated cells which have recently been described in the distal convoluted tubule of the rat (Griffith et al, 1968) and man (Tisher et al, 1968) are also present within the distal convoluted tubule of the rhesus monkey kidney (Tisher, unpublished observations). There is a gradual transition from the distal convoluted tubule to the cortical collecting duct.



Figure 34. HIGH MAGNIFICATION ELECTRON MICROGRAPH OF A MYOEPITHELIOID CELL CONTAINING THE RECTANGULAR OR OBLONG SECRETORY GRANULES BELIEVED TO BE RENIN. Many granules have a central density with a crystalline substructure of approximately 80 angstrom periodicity surrounded by less dense material. X 27,800

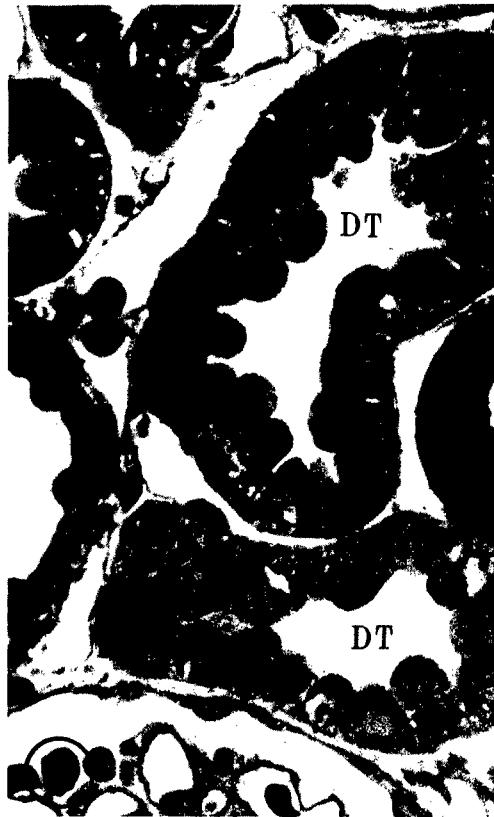


Figure 35. PHOTOMICROGRAPH OF DISTAL CONVOLUTED TUBULES (DT) FROM RHESUS MONKEY KIDNEY. X 600

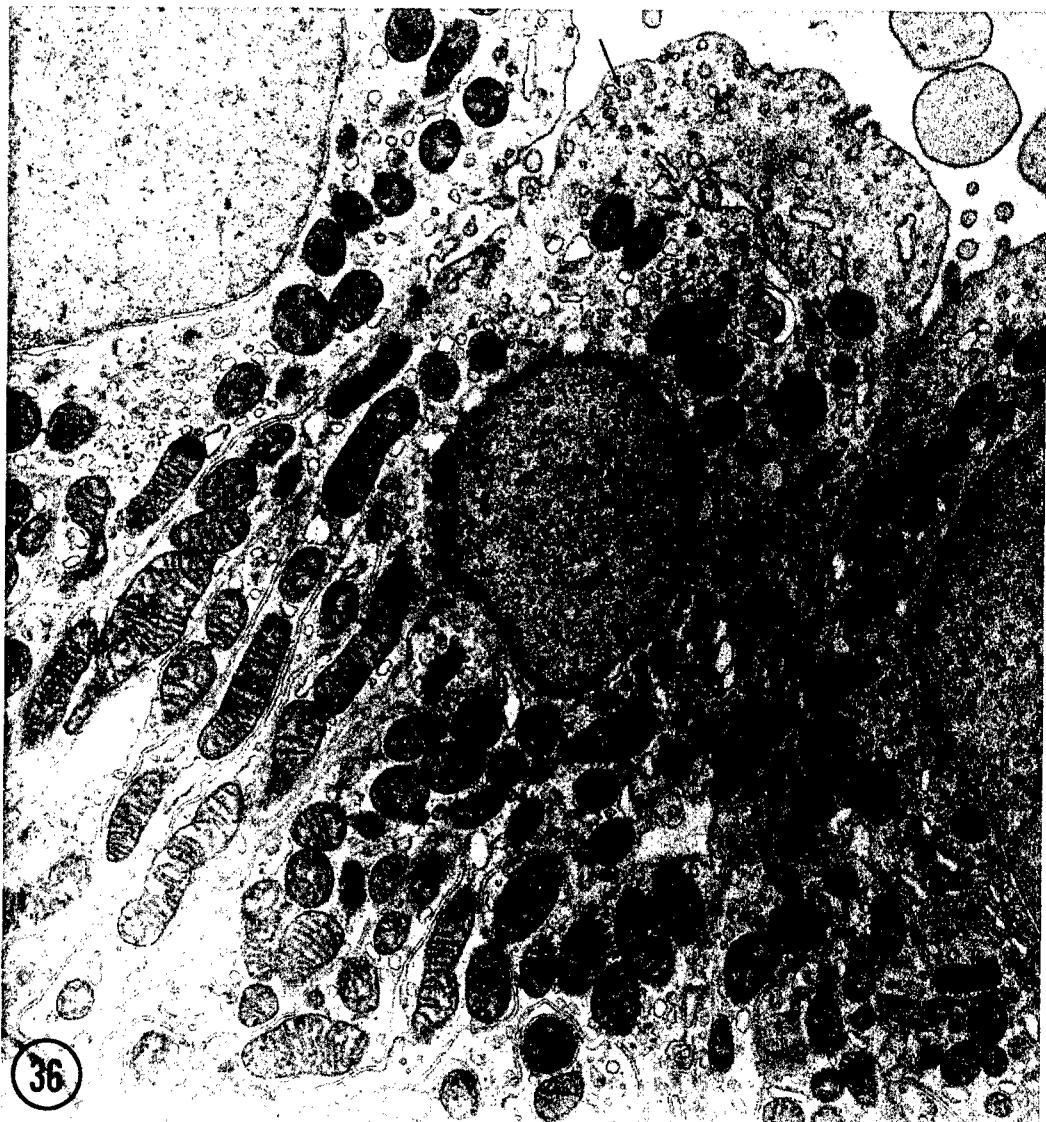


Figure 36. ELECTRON MICROGRAPH OF A DISTAL CONVOLUTED TUBULE. The cells are filled with elongate mitochondria (M), many enclosed within plications of the basal plasmalemma. Numerous vesicles are apparent on the apical cell surface (arrow). X 9,000

For purposes of organization, the collecting duct can be divided into four segments based primarily on their location in the kidney. The initial segment, the cortical collecting tubule or connecting segment, extends from its transition with the distal convoluted tubule to the medullary ray. The three remaining segments include the collecting tubule in the medullary ray, the outer medullary segment, and the inner medullary segment. With the exception of the inner medullary segment, the structural characteristics of the collecting duct of the rhesus monkey are nearly identical to that of the human kidney. The inner medullary segment is extremely abbreviated in length, however, compared to man. As the collecting ducts of the outer medulla descend toward the inner medulla they join very quickly to form the ducts of Bellini. These ducts are lined by a modified type of transitional epithelium, identical in appearance to the epithelium overlying the tip of the renal papilla (Tisher, in press) (figure 37). The collecting duct or tubule contains two types of cells, the light cell which is the principal cell type and the "intercalated" or dark cell (figure 38). The latter are most abundant in the cortical collecting tubule and decrease in frequency as the collecting duct descends into the inner medulla. In the inner medulla only light cells are present. The light cells contain a rather clear cytoplasm, a centrally-placed nucleus, and small numbers of randomly oriented mitochondria (figure 39). The dark cells possess a much darker cytoplasm due to the presence of increased numbers of cellular organelles including mitochondria, lysosomes, and granulated endoplasmic reticulum (figure 40). As in the human, the individual cells of the collecting duct progressively increase in height as the duct descends into the inner medullary region. With the exception of the inner medullary segment, the fine structural characteristics of the rhesus monkey collecting duct closely parallel those of man.

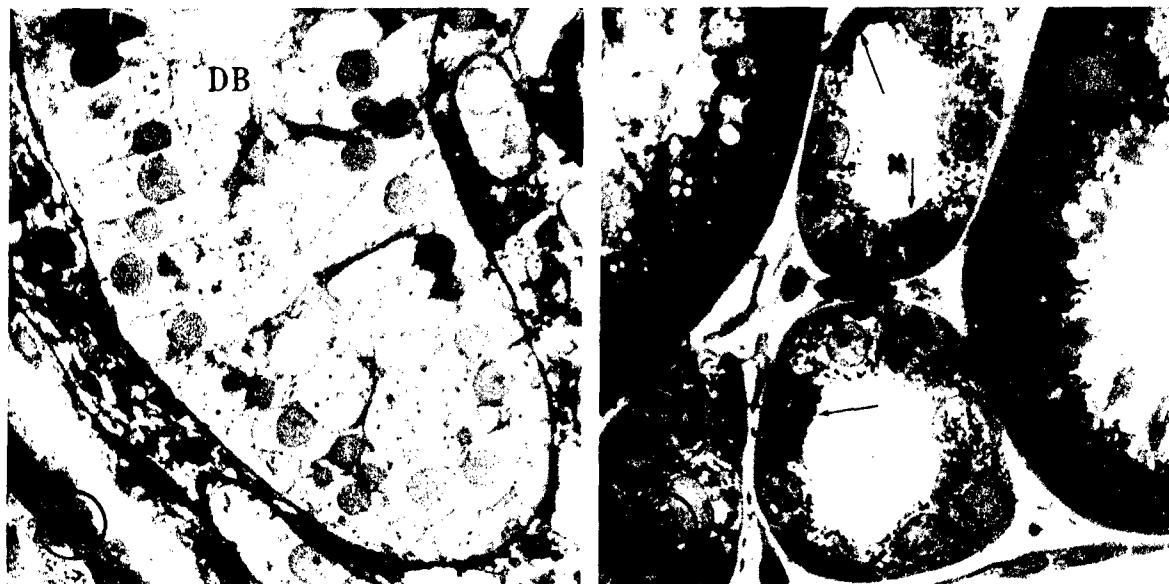


Figure 37. PHOTOMICROGRAPH SHOWING THE DUCT OF BELLINI (DB) IN THE INNER MEDULLA NEAR THE PAPILLARY TIP OF A NON-DISEASED RHESUS MONKEY KIDNEY. X 150

Figure 38. PHOTOMICROGRAPH SHOWING THE DIFFERENCES IN STRUCTURAL APPEARANCE BETWEEN DARK OR INTERCALATED CELLS (ARROWS) AND THE LIGHT OR PALE CELLS IN THE CORTICAL COLLECTING TUBULE.  
X 900



Figure 39. AN ELECTRON MICROGRAPH SHOWING THE TYPICAL APPEARANCE OF LIGHT OR PALE CELLS IN THE CORTICAL COLLECTING TUBULE. Note the paucity of organelles and the random distribution of mitochondria throughout the cell cytoplasm. X 15,750

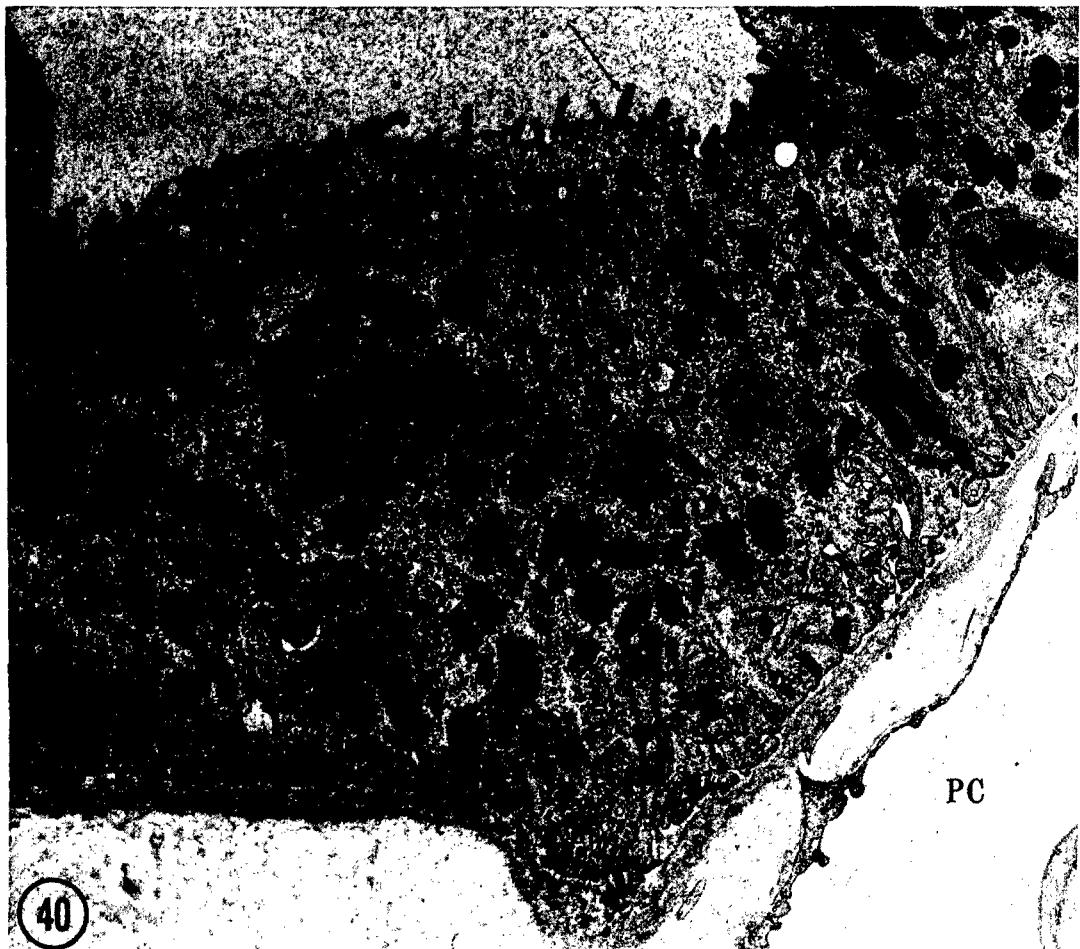


Figure 40. ELECTRON MICROGRAPH OF A DARK OR INTERCALATED CELL FROM THE CORTICAL COLLECTING TUBULE. The cytoplasm is very dense and contains large numbers of free ribosomes and clusters of rough surfaced endoplasmic reticulum, cytosomes, and increased numbers of mitochondria. Small microvilli are present on the apical surface of the cell (arrow). The basal plasmalemma is much more complex in configuration than that of light cells within this segment of the nephron. PC, peritubular capillary. X 9,240

In summary, gross, light and electron microscopic morphological observations of the kidney of the non-diseased rhesus monkey have been presented. Where pertinent, certain parameters of renal function have been reviewed and appropriate correlations made with the structure of the kidney. At the ultrastructural level, emphasis has been placed on many morphological variations in the kidney that have been observed in these apparently non-diseased animals. In part, these variations appear to represent biological differences among individual animals exposed to a "hostile" environment. Finally, morphological variations that can be ascribed to the method of tissue preservation that has been employed have been stressed.

#### ACKNOWLEDGEMENTS

The author gratefully acknowledges the valuable contributions of several colleagues involved in various aspects of the work reported in the present paper. These individuals include Drs. Seymour Rosen, Richard M. Finkel, Paul E. Teschan, and R. W. Schrier. Permission was granted by the editors of the American Journal of Pathology to reproduce figures 11-20, 23-25, and 29, and tables I and II from a previous publication by the author (Tisher et al, 1969). Similar permission was granted by the editors of Laboratory Investigation to reproduce figures 6, 8, 10, 21, 22, and 33 from previous publications by the author (Rosen and Tisher, 1968; Tisher et al, 1968). Figures 31 and 36 were kindly supplied by Dr. Seymour Rosen to whom the author is greatly indebted.

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## DISCUSSION

DR. BEARD: Dr. Tisher, I'm greatly impressed with the beauty of your pictures. I'm not a pathologist, I don't know too much about this but one point which I hope you'll bring out in publishing this is what you mean by healthy and normal. I presume these animals had their share of intestinal parasites.

DR. TISHER: Yes, that certainly is true, but by the clinical tests that are commonly employed, with particular stress on renal function tests, these animals were not clinically diseased. To give you an example, we measured electrolytes repeatedly, looked at urinary sediment, measured the endogenous creatinine clearances, and they also had a full battery of liver enzymes. We recognized the fact that they could have intestinal parasites. They were routinely checked and when this was confirmed, they were treated.

DR. PECK (Merck Institute for Therapeutic Research): I greatly appreciate the information you've presented. This is one of our problems in the area of drug toxicity, what is the change due to the drug and the change due to normal physiological conditions, diuresis, or increased blood flow. How can we interpret these changes as being something which is pathological or something which is physiological. We need more publications like this to help us along.

## HEMATOLOGIC CHANGES AS INDICATORS OF TOXICITY

Marylou B. Ingram, M.D.

University of Rochester  
Rochester, New York

### INTRODUCTION

Dr. Hodge gave this conference a scholarly and timely send-off by reminding us that the first session, which was concerned with toxic effects of carbon monoxide, represented the application of some of civilization's newest technology to a truly ancient problem.

Although my subject has been of interest for only a couple of centuries it is still moderately venerable, for almost as soon as morphologic criteria for blood cell types were established, it was recognized that a variety of chemical and physical agents as well as infectious agents and disease states of undetermined cause tended to induce changes in the number and structure of circulating blood cells. In harmony with Dr. Hodge's keynote, I should like to point out that the practical significance of blood cell changes as indicators of toxicity can be enormously enhanced through the application of newer technology.

### BLOOD CELLS AS INDICATORS OF TOXICITY

It is not surprising that blood cell changes are good indicators of toxicity. Blood cells are continually being lost and replaced by means of well controlled processes that maintain levels of circulating blood cells within remarkably narrow limits under normal conditions, and yet permit prompt acceleration of production of one or more types of cells in response to suddenly increased demand. Maintaining a steady-state blood picture involves both cell proliferation and a complex process of differentiation. When the demand for cells is truly acute, the cells produced may be measurably different from their normal counterparts.

In short, blood cell changes are good indicators of toxicity because 1) as in the case of other rapidly proliferating tissue, the cellular constituents are sensitive to even low levels of noxious agents, 2) diffuse distribution of hematopoietic tissue increases the probability that even localized stimuli will reach some part of it, 3) non-fatal injury of precursor cells is perpetuated in the cells' progeny (i.e. it is amplified

through cell division), and 4) blood and hematopoietic tissue can be repeatedly sampled relatively easily and with little risk.

Remember also that hematopoietic tissue has a truly phenomenal capacity for recovery from injury. Thus, if one concentrates on identifying early and subtle changes in the blood cell picture, he can be reasonably confident that he is detecting effects that are reversible if exposure to the noxious agent under consideration is stopped. A corollary of this, of course, is that not all measurable variability represents "damage".

### CONTRIBUTIONS OF MODERN TECHNOLOGY

Modern technological advances have already made it possible to make accurate measurements of certain characteristics of blood cells that could not be measured accurately previously. The Coulter Counter, for example, not only revolutionized blood cell counting; it can also be used to measure the volumes of very large numbers of individual blood cells rapidly and automatically. This has provided new insight into the kinetics and control of blood cell production. In our laboratory, for example, we have found red cell age:volume:density relationships to be surprisingly sensitive indicators of altered red cell production (Coopersmith; Coopersmith, 1968). Yesterday, Dr. Leon described another instrument, the Fragiligraph, that provides elegant data relative to the susceptibility of red cells to lysis by hypotonic saline solutions and other lytic agents.

Automating microscopy is several orders of magnitude more complex than automating cell counting or measuring osmotic fragility. Nevertheless, the age of automatic image processing systems is here. The need for it in both clinical and research laboratories is probably obvious to most of you. Routine differential blood cell counting has attracted the attention of several manufacturers of scientific instruments, and automatic systems for performing this determination will be appearing in the near future. Although estimating the relative numbers of a few major classes of leukocytes may be approached in several ways, other variables that are usually evaluated by examining stained blood smears will probably require automatic analysis of conventional microscope images. Image processing techniques will almost certainly be required to obtain, automatically, accurate quantitative information about such morphological characteristics as number and size of nuclear lobes and vacuoles; presence, size, number, and shape of nucleoli; range of variability in cell size and shape; and so forth. It will also be required to determine the true rates of occurrence of certain rarely occurring types of cells such as nucleated red cells and "blasts". Table I lists some examples of anomalies in one type of leukocyte, the neutrophil granulocyte, and a brief reference to the significance of each. Similar tables could be constructed for other types of blood cells.

TABLE I  
SOME ANOMALIES OF NEUTROPHIL MORPHOLOGY

<u>Structure</u>	<u>Significance</u>
Sex chromatin appendage (nuclear)	genetically female
Pelger-Huet anomaly (nuclear)	hereditary
May-Heggelin anomaly (cytoplasm)	hereditary
Döhle bodies (cytoplasm)	cell maturation defect
Macropolycytes, familial (giant, hypersegmented)	hereditary
Macropolycytes, nonfamilial (hypersegmentation)	megaloblastic anemia
Multiple cytoplasmic vacuoles, degranulation	bacteremia
Hyposegmentation of nuclei ("left shift")	acute infection, inflammation
Many coarse, basophilic ("toxic") granules	acute infection, inflammation
Numerous thread-like chromatin appendages	high correlation with cancer
Malignancy-associated changes (chromatin structure)	high correlation with cancer

For a number of years I have been collaborating with a group of engineers in the Electro-optical Research Division of the Perkin-Elmer Corporation in their development of a practical laboratory instrument for the analysis of blood cell images. We now have a system which can automatically scan a stained blood film, stop at leukocytes (rejecting platelets, artifacts such as dirt or stain precipitates), complete a highly sophisticated analysis of the leukocyte images and print out the cell types. It also provides numerical data describing many of the morphological characteristics referred to above. This system is called CELLSCAN-GLOPR<sup>®</sup> (GLOPR for Golay logic processor). A fairly complete description of the system has been written up for publication and is in press, but a brief description is appropriate here (3).

The CELLSCAN-GLOPR television microscope uses an oscillating mirror scanner to scan the image formed by the microscope. The image is converted into electronic signals by a photomultiplier tube. The converted image can then be displayed for visual

examination or processed by the CELLS CAN-GLOPR system. For computer processing, the microscope image is converted to a binary (black and white) image, a process referred to as "cartooning", and the number of image points is reduced from 40,000 (200 x 200) image points in the  $20\mu x 20\mu$  microscope field of view to 128 x 128, 64 x 64, or 32 x 32 points. In analyzing the image, the computer first forms a spectro-photometric histogram of the image formed at a selected wave length of illumination. This histogram represents the frequency distribution of photometric values of all image points. Characteristically there are three peaks corresponding to nucleus, cytoplasm, and background. Three cartoons are then formed by selecting quantizing levels the correspond to 1) the nuclear peak, 2) the valley between the nuclear and red cell peaks, and 3) the red cell peak. The three cartoons are then processed simultaneously by means of a series of Golay hexagonal pattern transforms which analyze each image point by comparing it with the binary state of the nearest neighbor points surrounding it in a hexagonal array. The many logic operations that can be performed with the special purpose CELLS CAN-GLOPR computer can be applied in making measurements of various topological features such as total area of the cell or its nucleus, calculating the length of the periphery of a cell or other image constituents, analyzing and quantitating fine structure, identifying and measuring concavities, nuclear lobes, and so forth. These numerical data are then analyzed to arrive at a cell classification.

At present I am on sabbatical leave at Jet Propulsion Laboratory, California Institute of Technology, participating in research on image processing with ALMS (automatic light microscope system). This program utilizes a large general purpose computer facility and can call upon the remarkably extensive software (to say nothing of the expertise in image processing) developed for the space program. We are concentrating first on the automation of karyotyping, another procedure for which automatic methods are urgently needed. Although the work on automatic karyotyping at JPL has been in progress for only a few months, Dr. Kenneth R. Castleman, Dr. Robert Nathan and others in the Image Processing group at JPL have already made some major advances. Since karyotyping is usually done on metaphase spreads of peripheral blood lymphocytes that have been cultured in vitro with PHA, it may be considered as another example of the way in which blood cells may provide information about the toxicity of various agents.

## CONCLUSION

The toxicologist does not have available to him very many cellular systems that are both highly responsive to a variety of toxic agents and easily accessible for serial sampling. Blood, however, is such a tissue.

Modern technological developments such as instruments for counting and sizing blood cells, measuring resistance to hemolysis by hypotonic solutions and other lytic agents and automatic image analysis systems that permit accurate measurement of subtle morphological changes may be expected to enhance the significance of blood cell changes as indicators of toxicity.

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## A CLINICAL EVALUATION OF THE HEMATOLOGIC AND BLOOD CHEMICAL PROFILES OF PRIMATES

Arthur S. Hall, D.V.M., M.S.

Oregon Regional Primate Research Center  
Beaverton, Oregon

### INTRODUCTION

Biological information gathered from non-human primates can be more meaningful to human biology than that obtained from other mammals. In recent years, the primate has become more useful in biomedical research. As scientific investigations continue, the quality of animals has declined.

To purchase large numbers of healthy, wild-caught animals is difficult today. The use of wild-caught monkeys presents the scientist with uncertain health, unknown genetic, and clinical difficulties. Many monkeys received from importers must be treated for months before they are adequate subjects for medical research. Some of the animals that survive may be poor animal models for research. Research data cannot be obtained without the knowledge of the normal biological properties of the species to be used in the investigation. The clinical management of several thousand primates which have been studied through hundreds of experiments over a period of six years has demonstrated many factors which alter the normal resting individual's clinical profile. The normal animal in special circumstances sometimes presents different clinical profiles. This presentation will evaluate the hematological and blood chemical data in relation to factors that may change the normal resting profile in monkeys.

### MATERIALS AND METHODS

The monkeys in this study were considered normal by general appearance and not by the clinical data from the blood samples. All the normal monkeys in this summary had completed a 90-day quarantine and conditioning period. If more than one normal determination was recorded for any individual test, the last value recorded was used to represent the normal value for that monkey. The number of monkeys listed for each test represents separate monkeys. Blood samples were usually collected in the morning hours before feeding time. Most of the blood samples were collected from the saphenous vein. For the collection of blood samples, the monkeys were restrained by caretakers with heavy leather gloves. The animals were fed once daily a commercial monkey chow

(Purina Monkey Chow, Ralston Purina Co., St. Louis, Missouri) with an apple supplement three times a week. Monkeys were kept in single cages and in gang cages. For each chemical determination, the data were separated by sex, method of determination, genus, and species of monkey. Not all of the categories were compared and discussed in this report.

The clinical information was stored and analyzed by an XDS 920 computer, which used a medical records information system (P. R. I. M. E.), (Lusted, 1967). This system allows the data to be retrieved and arrayed in a Fortran-compatible format. The normal monkey values were selected from more than 5000 separate clinical records. These data were accumulated for a period of six years. The normal monkeys were sampled in the routine clinical testing procedures of the primate colony. Five technicians assisted in the collection and the determination of these values.

The hematological and blood chemical values were measured according to the following procedures:

Red Blood Cell Counts. A 1:50,000 blood dilution in saline was electronically counted. (Model B Coulter Counter, Coulter Electronics, Hialeah, Florida.)

Hemoglobins. The optical density of a 1:250 dilution of blood and Drabkins solution was measured using a 540  $\mu$  filter on a colorimeter. (Klett-Summerson, Colorimeter, Klett, Manufacturing Company, New York, New York.)

Packed Cell Volumes. Microhematocrit tubes were filled with whole blood and centrifuged for approximately 5 minutes in a microhematocrit centrifuge. (Phillips-Drucker Microhematocrit Centrifuge, Astoria, Oregon.)

White Blood Cell Counts. A 1:500 blood dilution was added to a saponin solution. After red blood cell hemolysis, the WBC solution was electronically counted. (Model B Coulter Counter, Coulter Electronics, Hialeah, Florida.)

Differential White Blood Cell Counts. Blood smears were stained 3 minutes with Wright stain and rinsed 8 minutes with a buffered solution.

Platelet Counts. A 1:100 dilution of oxalated blood was observed on a hemocytometer for the platelet count. (Brilliant Cresyl Blue stain).

Blood urea nitrogen was determined by the Berthelot reaction using urease (Chaney and Marbach, 1962). Total serum protein was determined by the biuret procedure (Kingsley, 1939). Albumin and globulin were assayed by cellulose acetate electrophoresis (Grunbaum et al, 1960). Sodium and potassium were performed using flame photometry (Hald et al, 1958). Chlorides were determined by mercuric nitrate titration (Schales, 1944). Serum glucose was determined by a photometric method of Nelson (1944) on all the monkeys except the M. fuscata. The M. fuscata glucose assays were performed by an enzymatic reaction (Stein, 1963). Total plasma cholesterol was performed by methods described by Abell (1953) and Zak (1954). Calcium was measured by EDTA

titration using calcein as an indicator (Bachra et al, 1958). *M. mulatta* serum calciums were determined by a photometric method (Ferro and Ham, 1958). Phosphorus values were assayed by a method described by Dryer (1957). Alkaline and acid phosphatase were determined by the Bodansky method (1933) using  $\beta$ -glycerophosphate as a substrate. Serum glutamic pyruvic transaminase, serum glutamic oxalacetic transaminase, and lactic dehydrogenase were assayed by commercially available reagents (Henry et al, 1960). Arterial pH,  $\text{pCO}_2$ , and  $\text{pO}_2$  were determined by the Astrup (1959) method.

The uric acid method was described by Henry (1957). The bilirubin method performed on the adult *M. mulatta* was described by Malloy (1937). The infant bilirubin data was obtained by the method of Powell (1944). The creatinine method was described by Bonsnes (1945).

## RESULTS

### Red Blood Cell Counts

TABLE I  
STATISTICAL SUMMARY

SPECIES	Clinical Determination: Red Blood Cells				NUMBER OF ANIMALS			
	Method ( $\pm 2$ ) STD DEV:		$(\pm 1)$ STD DEV					
	♂	♀	♂	♀				
<i>Macaca mulatta</i>	5.3	5.4	.6	.7	66	248		
<i>Macaca niger</i>	5.8	5.7	.5	1.0	5	14		
<i>Macaca nemestrina</i>	6.0	5.7	.5	.3	7	20		
<i>Macaca fuscata</i>	4.6	4.6	.5	.4	33	38		
<i>Macaca speciosa</i>	5.3	5.2	.2	.4	7	3		
<i>Saimiri sciurea</i>	-	7.0	-	.5	-	3		
<i>Macaca fascicularis</i>	-	5.1	-	.7	-	51		
<i>Galago crassicaudata</i>	6.8	6.1	1.3	1.2	36	21		
<i>Lemur fulvus</i>	8.0	8.3	.9	.9	13	17		
<i>Lemur catta</i>	7.2	7.0	.3	.5	11	9		

Anemias can result from either decreased red cell production, increased red cell destruction, or blood loss.

In a primate colony, anemia from blood loss is common, massive hemorrhage from lacerated sex flesh and long menstrual cycles being common among females. Chronic menorrhagia will usually yield a decreased hematocrit and hemoglobin from the normal values (table II and table III). Hemorrhage into the gastrointestinal tract from ulcerations due to shigellosis is frequently encountered in male and female monkeys.

TABLE II  
STATISTICAL SUMMARY

Clinical Determination: Hemoglobin

Method ( $\pm$  2) STD DEV: ( $\pm$  .4 Gms/100 ml.)

Method: Cyanmethemoglobin

Units: Gms/100 ml

SPECIES	MEAN		(+ 1) STD DEV		NUMBER OF ANIMALS	
	<u>♂</u>	<u>♀</u>	<u>♂</u>	<u>♀</u>	<u>♂</u>	<u>♀</u>
<u>Macaca mulatta</u>	12.9	12.6	1.4	1.5	169	581
<u>Macaca niger</u>	11.4	10.6	1.7	.7	7	16
<u>Macaca nemestrina</u>	12.1	11.1	1.4	1.2	19	33
<u>Macaca fuscata</u>	13.0	13.5	1.1	1.0	39	38
<u>Macaca speciosa</u>	12.1	12.7	1.3	1.1	12	4
<u>Saimiri sciurea</u>	-	11.9	-	1.0	-	72
<u>Macaca fascicularis</u>	-	10.8	-	1.2	-	58
<u>Galago crassicaudata</u>	16.2	14.9	1.5	1.4	34	24
<u>Lemur fulvus</u>	14.0	14.0	1.7	1.3	14	19
<u>Lemur catta</u>	15.5	15.1	1.2	1.1	11	9

TABLE III  
STATISTICAL SUMMARY

SPECIES	Units: Percent (%)				NUMBER OF ANIMALS	
	MEAN <u><math>\bar{x}</math></u>	STD DEV <u><math>\pm</math></u> <u><math>\sigma</math></u>	( $\pm$ 1) <u><math>\pm</math></u> <u><math>\sigma</math></u>	STD DEV <u><math>\pm</math></u> <u><math>\sigma</math></u>	<u><math>\bar{x}</math></u>	<u><math>\sigma</math></u>
<u>Macaca mulatta</u>	42.5	41.8	4.2	4.3	169	546
<u>Macaca niger</u>	41.1	39.6	3.4	2.1	7	17
<u>Macaca nemestrina</u>	44.2	39.9	3.1	4.2	19	33
<u>Macaca fuscata</u>	40.8	41.4	3.0	3.3	39	36
<u>Macaca speciosa</u>	40.6	42.3	3.1	3.0	12	4
<u>Saimiri sciureus</u>	-	40.5	-	2.9	-	72
<u>Macaca fascicularis</u>	-	39.8	-	3.6	-	58
<u>Galago crassicaudata</u>	49.1	45.2	4.8	4.2	37	29
<u>Lemur fulvus</u>	42.6	42.8	4.5	3.0	14	19
<u>Lemur catta</u>	50.4	49.2	3.8	2.9	11	9

When platelet counts fall below 1,000 platelets/cu mm massive hemorrhage will occur in many organs of the body. Normal platelet counts have ranged from 150,000 to 500,000 cu mm in this primate colony. South American monkeys have malarial and microfilarial infections which could be troublesome to chronic studies involving hematological profiles. Prosimians have consistently higher packed cell volumes, hemoglobin, and red blood cell counts than the Macaca sp. Packed cell volumes vary with the posture of monkeys and methods of restraint. Rhesus monkeys restrained in chairs maintain high hematocrits. (If blood samples are collected from femoral and saphenous veins).

Total White Blood Cell and Differential Cell CountsTABLE IV  
STATISTICAL SUMMARYClinical Determination: White Blood CellsMethod ( $\pm$  2) STD DEV: ( $\pm 0.6 \times 10^3$  cmm)Method: Coulter CounterUnits:  $10^3$  cmm

<u>SPECIES</u>	<u>MEAN</u>		<u>(<math>\pm</math> 1) STD DEV</u>		<u>NUMBER OF ANIMALS</u>	
	<u>♂</u>	<u>♀</u>	<u>♂</u>	<u>♀</u>	<u>♂</u>	<u>♀</u>
<u>Macaca mulatta</u>	10.9	12.1	4.1	4.6	161	575
<u>Macaca niger</u>	14.7	16.3	4.4	5.9	7	17
<u>Macaca nemestrina</u>	10.8	11.6	3.2	4.5	19	33
<u>Macaca fuscata</u>	15.0	14.2	5.0	5.6	42	43
<u>Macaca speciosa</u>	15.9	12.5	3.7	1.7	12	4
<u>Saimiri sciurea</u>	-	12.4	-	5.3	-	72
<u>Macaca fascicularis</u>	-	16.9	-	6.2	-	58
<u>Galago crassicaudata</u>	11.0	9.5	4.5	2.3	34	21
<u>Lemur fulvus</u>	10.0	9.6	3.8	3.0	14	19
<u>Lemur catta</u>	8.1	8.7	2.5	2.9	11	9

TABLE V  
STATISTICAL SUMMARY

<u>SPECIES</u>	<u>Units: Per cent (%)</u>				<u>NUMBER OF ANIMALS</u>	
	<u>♂</u>	<u>MEAN ♀</u>	<u>(+ 1) STD DEV</u>		<u>♂</u>	<u>♀</u>
<u>Macaca mulatta</u>	42.1	37.7	19.6	16.5	158	493
<u>Macaca niger</u>	26.1	34.3	14.8	9.1	7	16
<u>Macaca nemestrina</u>	38.0	40.4	13.3	15.4	19	31
<u>Macaca fuscata</u>	46.5	61.8	22.7	31.9	10	5
<u>Macaca speciosa</u>	35.5	27.0	17.0	12.7	12	4
<u>Saimiri sciurea</u>	-	37.8	-	14.3	-	73
<u>Macaca fascicularis</u>	-	40.5	-	14.2	-	58
<u>Galago crassicaudata</u>	27.8	22.6	10.6	8.8	33	22
<u>Lemur fulvus</u>	28.6	32.1	15.2	17.2	14	19
<u>Lemur catta</u>	33.8	28.4	11.6	9.3	11	9

TABLE VI  
STATISTICAL SUMMARY

<u>SPECIES</u>	<u>Clinical Determination:</u>		<u>Lymphocytes</u>		<u>(+ 1)</u>		<u>NUMBER OF ANIMALS</u>	
	<u>MEAN</u>	<u>♂</u>	<u>MEAN</u>	<u>♀</u>	<u>STD DEV</u>	<u>♂</u>	<u>♀</u>	<u>♂</u>
<u>Macaca mulatta</u>	52.1		55.1		20.3	18.5		154 491
<u>Macaca niger</u>	63.7		54.8		20.0	11.7		7 16
<u>Macaca nemestrina</u>	55.2		50.9		15.3	16.0		19 33
<u>Macaca fuscata</u>	47.7		33.8		23.4	30.8		10 5
<u>Macaca speciosa</u>	55.9		64.8		18.4	15.4		12 4
<u>Saimiri sciurea</u>	-		55.4		-	13.1		- 72
<u>Macaca fascicularis</u>	-		42.0		-	13.5		- 57
<u>Galago crassicaudata</u>	65.6		70.1		11.4	10.7		33 22
<u>Lemur fulvus</u>	61.1		53.2		18.8	17.3		14 19
<u>Lemur catta</u>	57.3		60.0		12.7	11.7		11 9

Apparently exercise and excitement stimulate leukocytosis. White cell counts vary greatly in primates caged in large compounds from monkeys caged in single cages. Another factor that elevates the white blood count is the small laceration from bite wounds that are commonplace in monkeys caged with one or more monkeys. In long term studies, tartar accumulation on teeth and gingivitis are continual problems that elevate the white blood cell counts. In large groups, the least dominant monkeys often have severe bite wounds and muscle contusions which alter the biochemical and hematological assays. Without physical examinations, the hematological evaluations of a study may be misleading.

Healthy primates usually exhibit a 60% lymphocyte count with 30% mature neutrophil counts. During a 90-day conditioning period white blood cell counts can be expected to be higher at the beginning of the period than the end of the conditioning period.

Bacterial infections such as shigellosis or pneumonia cause a leukocytosis with an increased number of immature and mature neutrophils.

Minor lacerations or small local infections usually result in a shift in the differential cell count to 60% neutrophils with 30% lymphocytes without a large increase in the total white blood count.

Eosinophil counts may be increased in severe lung mite infestations or other parasite infections.

### Blood Urea Nitrogen

TABLE VII  
STATISTICAL SUMMARY

SPECIES	Clinical Determination: Urea Nitrogen				NURBS OF ANIMALS	
	Method: Berthelot Reaction					
	Units: mg/100ml					
	MEAN	(± 1) STD DEV	(± 2) STD DEV			
	$\bar{x}$	$\bar{x} \pm 1$	$\bar{x} \pm 2$	$\bar{x}$	$\bar{x}$	
<u>Macaca mulatta</u>	18.6	18.1	4.4	6.5	37	
<u>Macaca nigra</u>	15.1	18.9	4.1	4.0	6	
<u>Macaca nemestrina</u>	19.6	15.3	2.4	4.3	5	
<u>Macaca fuscata</u>	20.7	20.0	3.1	3.2	64	
<u>Macaca speciosa</u>	18.3	16.9	4.4	4.8	6	
<u>Saimiri sciureus</u>	-	26.1	-	4.1	-	
<u>Macaca fascicularis</u>	-	21.3	-	12.8	-	
<u>Galago crassicaudata</u>	21.3	19.6	3.5	3.9	30	
<u>Lemur fulvus</u>	18.7	17.8	5.6	2.4	3	
<u>Lemur catta</u>	12.4	15.5	.3	1.6	2	

Blood urea nitrogen may be expected to vary with dietary protein intake and illness. Blood creatinine, being independent of exogenous nitrogen and being related to body muscle mass, should seldom change in the normal monkey. Squirrel monkeys with continuous feedings of 25% protein exhibit a high BUN. Macaca fuscata fed 25% protein diets have a higher BUN value than Macaca mulatta fed 15% protein diets. Starvation and febrile disorders, conditions commonly seen in monkeys in outdoor gang cages, produced protein catabolism with an increase in blood urea nitrogen. Hypovolemic shock created by diarrhea or hemorrhage decreases the renal blood flow. With decreased renal blood flow, blood urea nitrogen and blood creatinine increase.

### Serum Proteins

TABLE VIII  
STATISTICAL SUMMARY

SPECIES	MEAN		(+ 1) STD DEV		NUMBER OF ANIMALS	
	<u>♂</u>	<u>♀</u>	<u>♂</u>	<u>♀</u>	<u>♂</u>	<u>♀</u>
<u>Macaca mulatta</u>	8.3	8.6	.9	1.0	68	311
<u>Macaca niger</u>	8.6	8.5	.9	.9	3	14
<u>Macaca nemestrina</u>	8.0	8.3	1.2	1.4	4	23
<u>Macaca fuscata</u>	8.0	8.2	1.0	1.2	21	19
<u>Macaca speciosa</u>	8.2	7.6	1.0	.3	4	3
<u>Saimiri sciurea</u>	8.8	8.8	.1	1.2	2	14
<u>Macaca fascicularis</u>		7.8		.4		2
<u>Galago crassicaudata</u>	7.8	7.8	.6	.6	8	3
<u>Lemur fulvus</u>	8.1	7.6	.0	1.1	1	3
<u>Lemur catta</u>	8.1	7.4	.2	.1	4	3

TABLE IX  
STATISTICAL SUMMARY

<u>SPECIES</u>	<u>Clinical Determination: Albumin</u>				<u>NUMBER OF ANIMALS</u>	
	<u>MEAN</u>		<u>(<math>\pm</math> 1) STD DEV</u>			
	<u><math>\delta</math></u>	<u><math>\varphi</math></u>	<u><math>\delta</math></u>	<u><math>\varphi</math></u>	<u><math>\delta</math></u>	<u><math>\varphi</math></u>
<u>Macaca mulatta</u>	3.7	4.5	1.0	.9	36	170
<u>Macaca niger</u>	4.7	4.9	1.8	.9	2	8
<u>Macaca nemestrina</u>		4.2		1.2		10
<u>Macaca fuscata</u>	3.5	4.1	.8	1.2	6	9
<u>Saimiri sciurea</u>	4.8	4.4	1.3	.9	2	6
<u>Macaca fascicularis</u>	-	2.8	-	.0	-	1
<u>Galago crassicaudata</u>	4.1	4.4	.6	.5	4	2
<u>Lemur catta</u>	5.4	5.1	.2	.1	4	2

In newly acquired monkeys, the albumin to globulin ratio is reversed while the total serum protein values are decreased. As the animals become conditioned to the new environment, the albumin to globulin ratio returns to normal and the total protein increases to normal.

Acute and chronic bacterial infections increase the production of globulins. With severe infections, a shift is observed in the albumin to globulin ratio. Most intestinal infections of protozoa and nematodes cause an increase in globulin and decrease in albumin with total serum protein decreasing slightly.

ElectrolytesTABLE X  
STATISTICAL SUMMARY

Clinical Determination: Sodium						
Method: (± 1) STD DEV: (± 2.0 mEq/L)						
Method: Flame Photometry						
Units: mEq/L						
SPECIES	MEAN	(± 1) STD DEV	(± 1) STD DEV	NUMBER OF ANIMALS		
	<u><math>\bar{x}</math></u>	<u><math>\pm S</math></u>	<u><math>\pm S</math></u>	<u><math>n</math></u>	<u><math>\bar{x}</math></u>	<u><math>\pm S</math></u>
<u>Macaca mulatta</u>	150.6	152.1	9.3	9.2	60	256
<u>Macaca niger</u>	149.6	149.8	14.9	12.6	12	26
<u>Macaca nemestrina</u>	152.2	147.6	1.8	8.2	5	27
<u>Macaca fuscata</u>	157.4	151.5	11.0	10.8	24	16
<u>Macaca speciosa</u>	157.7	160.0	5.5	5.7	3	2
<u>Scinirri sciurea</u>	157.8	-	5.0	-	13	-
<u>Macaca fascicularis</u>	-	149.3	-	3.2	-	4
<u>Galago crassicaudata</u>	152.0	144.0	2.8	11.3	2	2
<u>Lemur fulvus</u>	156.7	160.5	5.8	12.0	3	2
<u>Lemur catta</u>	164.0	-	.0	-	1	-

TABLE XI  
STATISTICAL SUMMARY

Clinical Determination: Potassium						
Method: (± 3) STD DEV: (± 0.1 mEq/L)						
Method: Flame Photometry						
Units: mEq/L						
SPECIES	MEAN	(± 1) STD DEV	(± 3) STD DEV	NUMBER OF ANIMALS		
	<u><math>\bar{x}</math></u>	<u><math>\pm S</math></u>	<u><math>\pm 3S</math></u>	<u><math>n</math></u>	<u><math>\bar{x}</math></u>	<u><math>\pm S</math></u>
<u>Macaca mulatta</u>	4.4	4.3	.7	.9	60	252
<u>Macaca niger</u>	4.3	4.0	.7	.5	12	26
<u>Macaca nemestrina</u>	4.0	4.2	.4	.6	5	27
<u>Macaca fuscata</u>	4.3	3.9	.5	.6	23	16
<u>Macaca speciosa</u>	4.1	4.3	.3	.9	3	2
<u>Scinirri sciurea</u>	5.2	-	.8	-	13	-
<u>Macaca fascicularis</u>	-	6.0	-	1.0	-	4
<u>Galago crassicaudata</u>	5.2	4.6	.3	.0	2	1
<u>Lemur fulvus</u>	6.9	7.1	.4	.5	3	2
<u>Lemur catta</u>	6.5	-	.0	-	1	-

TABLE XII  
STATISTICAL SUMMARY

SPECIES	MEAN		$(\pm 1)$ STD DEV		NUMBER OF ANIMALS	
	$\sigma$	$\bar{x}$	$\sigma$	$\bar{x}$	$\sigma$	$\bar{x}$
<u>Macaca mulatta</u>	111.3	112.5	4.7	6.3	50	212
<u>Macaca niger</u>	115.0	111.0	4.2	2.6	2	3
<u>Macaca nemestrina</u>	112.6	111.3	5.7	4.7	10	26
<u>Macaca fuscata</u>	107.5	109.8	8.0	7.9	20	17
<u>Macaca speciosa</u>	103.3	109.0	1.2	1.4	3	2
<u>Saimiri sciurea</u>	123.4	-	4.5	-	36	-
<u>Macaca fascicularis</u>	-	115.0	-	5.6	-	4
<u>Lemur fulvus</u>	109.4	111.3	4.8	4.6	7	6
<u>Lemur catta</u>	112.7	113.0	.6	1.4	3	2

Dehydration from decreased oral intake and diarrhea depletes the intracellular and extracellular water store. In severe diarrhea, all serum electrolytes may be decreased. In starvation with decreased oral fluids intake, little or no change in serum electrolytes may be seen. Acute renal failure following diarrhea or trauma stops the kidney functions. The excretion of electrolytes is stopped. All the electrolytes usually increase; however, sodium may decrease with an increase in serum potassium and chloride only. Following muscular trauma or necrosis serum potassium may rapidly increase. Excitement in some squirrel monkeys causes a critical rise in serum potassium.

Serum Glucose and CholesterolTABLE XIII  
STATISTICAL SUMMARY

Clinical Determination: Glucose  
Method (+ 1) STD DEV: ( $\pm$  4.0 mg/100 ml)  
Method: Somogyi-Nelson  
Units: mg/100 ml

SPECIES	MEAN		( $\pm$ 1) STD DEV		NUMBER OF ANIMALS	
	$\sigma$	$\sigma$	$\sigma$	$\sigma$	$\sigma$	$\sigma$
<u>Macaca mulatta</u>	80.4	85.7	22.6	30.4	58	281
<u>Macaca niger</u>	82.1	104.2	39.7	39.8	14	26
<u>Macaca nemestrina</u>	61.2	78.8	19.0	28.1	13	28
<u>Macaca fuscata</u>	113.4	108.7	45.8	36.8	31	27
<u>Macaca speciosa</u>	55.6	56.5	6.6	16.8	5	6
<u>Saimiri sciurea</u>	91.3	125.0	12.9	57.2	3	64
<u>Galago crassicaudata</u>	205.9	207.0	62.7	45.7	20	21
<u>Lemur fulvus</u>		310.0	-	.0	-	1

TABLE XIV  
STATISTICAL SUMMARY

Clinical Determination: Cholesterol  
Method: Abell\* and Zak\*\*  
Units: mg/100 ml

SPECIES	MEAN		( $\pm$ 1) STD DEV		NUMBER OF ANIMALS	
	$\sigma$	$\sigma$	$\sigma$	$\sigma$	$\sigma$	$\sigma$
<u>Macaca mulatta**</u>	121	148	25	36	7	82
<u>Macaca niger**</u>	-	134	-	29	-	16
<u>Macaca fuscata*</u>	144	162	37	51	11	9

The most common condition which lowers blood glucose in monkeys is malnutrition. When monkeys are caged in groups, the timid monkeys of the group may fail to eat an adequate proportion of daily diet. On the other hand, the exercise and excitement of capture causes a marked increase in fasting blood glucose levels in the normal adult males. Overnight fasting is not adequate fasting time to prevent the epinephrine provoked glycogenolysis in the liver and muscles.

Primates caged in groups may vary markedly in their serum cholesterol values. Early starvation may cause increased cholesterol because of fats being mobilized from the fat stores. Malnutrition will decrease serum lipids because of the total depletion of the fat stores.

During pregnancy, cholesterol plasma values will usually decline until the delivery of the fetus in the rhesus monkey (Allen and Ahlgren, 1968).

#### Calcium, Phosphorus, and Alkaline Phosphatase

TABLE XV  
STATISTICAL SUMMARY

Clinical Determination: Calcium

Method: Calcein\*, and colorimetric\*\*

Units: mg/100 ml.

<u>SPECIES</u>	<u>MEAN</u>	<u>(+ 1) STD DEV</u>	<u>NUMBER OF ANIMALS</u>
<u>Macaca mulatta**</u>	9.2	1.06	28
<u>Saimiri sciurea*</u>	10.1	.93	144
<u>Galago crassicaudatta*</u>	10.6	.10	3
<u>Cebus albifrons</u>	10.7	1.08	11

TABLE XVI  
STATISTICAL SUMMARY

<u>SPECIES</u>	<u>MEAN</u>	<u>(<math>\pm</math> 1) STD DEV</u>	<u>NUMBER OF ANIMALS</u>
<u>Macaca mulatta</u>	5.2	1.5	198
<u>Macaca niger</u>	4.8	.9	13
<u>Macaca nemestrina</u>	3.9	1.7	8
<u>Macaca fuscata</u>	4.9	1.2	24
<u>Macaca speciosa</u>	5.6	2.7	4
<u>Saimiri sciureus</u>	3.4	.6	7
<u>Galago crassicaudata</u>	5.3	1.8	2
<u>Lemur fulvus</u>	6.4	2.0	2

TABLE XVII  
STATISTICAL SUMMARY

<u>SPECIES</u>	<u>MEAN</u>	<u>(<math>\pm</math> 1) STD DEV</u>	<u>NUMBER OF ANIMALS</u>
<u>Macaca mulatta</u>	21.5	18.5	44
<u>Macaca niger</u>	24.2	13.8	10
<u>Macaca nemestrina</u>	62.0	.0	1
<u>Macaca fuscata</u>	49.2	74.3	8
<u>Saimiri sciurea</u>	250.0	146.3	55

South American primates are susceptible to changes in the serum levels of calcium, phosphorus, and alkaline phosphatase. These primates must have vitamin D<sub>3</sub> in the diet to prevent osteomalacia. When monkeys are suffering from malnutrition, calcium and phosphorus will usually decrease causing an increase in alkaline phosphatase activity. Osteomalacia and parasitism which are common diseases of squirrel monkeys, cause the alkaline phosphatase activity to be variable and elevated.

Hypovitaminosis D<sub>3</sub> prevents the adequate absorption of calcium although the daily intake of calcium is normal. The serum calcium and phosphorus may be normal or decreased but serum alkaline phosphatase is elevated in hypovitaminosis D<sub>3</sub>.

### Serum Enzymes

TABLE XVIII

TEST	METHOD	SPECIES	MEAN	STD DEV	NUMBER
LDH	Worthington	<u>Lemur catta</u>	205.4	161.6	17
	"	<u>Lemur fulvus</u>	88.4	53.9	14
	"	<u>Galago crassicaudata</u>	295.6	190.0	50
SGPT	Worthington	<u>Macaca mulatta</u>	37.7	17.5	57
	"	<u>Lemur catta</u>	54.6	18.5	16
	"	<u>Lemur fulvus</u>	34.1	17.0	17
	"	<u>Galago crassicaudata</u>	18.9	8.1	47
	"	<u>Macaca nemestrina</u>	74.8	6.2	2
	"	<u>Macaca irus</u>	45.8	.0	1
SGOT	Worthington	<u>Macaca mulatta</u>	35.4	11.9	55
	"	<u>Lemur fulvus</u>	11.7	7.4	18
	"	<u>Galago crassicaudata</u>	24.7	10.4	49
	"	<u>Lemur catta</u>	20.3	4.2	9
	"	<u>Macaca irus</u>	33.4	.0	1

(A unit of enzyme activity is that causing a decrease in absorbancy of 0.001 per minute at 25°C.)

LDH, SGOT, SGPT, and CPK elevate quickly following trauma or surgical procedures. CPK activity parallels the transaminase activity closely.

Arterial pH, pO<sub>2</sub>, pCO<sub>2</sub>

TABLE XIX

TEST	METHOD	SPECIES	MEAN	STD DEV	NUMBER
pO <sub>2</sub> (ARTERIAL)	Astrup (mmHg.)	<u>Macaca mulatta</u>	96.4	9.7	29
pCO <sub>2</sub> (ARTERIAL)	Astrup (mmHg.)	<u>Macaca mulatta</u>	26.3	3.5	30
pH (ARTERIAL)	Astrup (units)	<u>Macaca mulatta</u>	7.4	.04	31

The use of chemical restraint to handle monkeys while monitoring the blood gases yields the best profile data. The excitement and exercise of physical restraint produces drastic changes in the acid-base balance. Squirrel monkeys may die while being restrained.

Lung mites must be considered in the evaluation of inhalation studies when wild-caught rhesus monkeys are the experimental animal. Radiographs should be taken to be sure pneumothorax has not developed.

Other Tests

Infants have elevated total serum bilirubin the first few days of life. These higher values are created by the presence of non-conjugated bilirubin in the serum.

TABLE XX

TEST	METHOD	SPECIES	MEAN	UNITS	NUMBER
ACID PHOSPHATASE	Bodansky	<u>Saimiri sciurea</u>	19.1	Shinowara	8
URIC ACID	Henry	<u>Macaca mulatta</u>	3.1	mg/100 ml	4
BILIRUBIN (Total)	Diazo	<u>Macaca mulatta</u>	.4	mg/100 ml	44 (Adults)
	"	<u>Macaca mulatta</u>	.7	mg/100 ml	24 (Infants)
CREATININE	Jaffe	<u>Macaca mulatta</u>	.7	mg/100 ml	4
	"	<u>Saimiri sciurea</u>	.4	mg/100 ml	1

## CONCLUSION

Psychological factors are important in the collection of monkey data. Noise of the infants from the nursery alters the menstrual cycles of the female breeding colony.

Male and female rhesus monkeys housed in single cages in the same room affects dietary intake which may be reflected in the resting profile. Young female Macaca nemestrina often lose weight and acquire a functional diarrhea when housed in a room with large adult males.

Animal conditions must be maintained from experiment to experiment. Cage designs must be similar and sex ratios in the same room must be constant.

Primates should have clinical profiles for individual animals. If an individual monkey profile is highly variable during the baseline study, the monkey should be reevaluated for use in the project.

Animals in limited confinement studies must be selected for temperament and disposition. Because individual monkeys vary psychologically, the best clinical data are derived from monkeys that have individual profiles compiled following an adequate conditioning time.

After the individual normal profiles are established, the experimental profiles may be collected.

An experienced team of primate handlers and technicians is one of the most important factors in collecting good profile data.

Routine weighing and tuberculosis testing are the most important procedures in the management of a primate colony. Periodic chest radiographs to supplement the tuberculin test add to the effectiveness of the testing program.

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## DISCUSSION

COLONEL STEINBERG (U.S. Army Environmental Hygiene Agency): Two questions, if you would. One, did you evaluate your primate data on the basis of age, in other words did you try a comparison of your younger monkeys against your older monkeys?

DR. HALL (Oregon Regional Primate Research Center): We have the data but the comparison hasn't been done yet. These are normals considering all ages.

COLONEL STEINBERG: Do you anticipate a difference?

DR. HALL: Well, as reported in white cell counts, I think that there are less differences than have been previously reported, but I would have to look at each individual test. The only one that I can think of that I questioned is the white cell count. For example, the BUN's seem to be about the same. Did you have one particular test in mind?

COLONEL STEINBERG: No, I'll go on to the second question. The other is on your Coulter Counter. Did you, on doing your WBC's and RBC's run into any trouble in regard to size of aperture that you used or what size aperture did you use?

DR. HALL: We used 100 micron and just lately they've come out with a reasonably good standard and, I didn't mention this, but the data was obtained by five different technicians over a period of six years and most studies of normal data you'll see are usually collected in a period of one year, six months with one technician. The data was collected on two Coulter Counters and comparing the two Coulter Counters it was identical by inspection. The aperture that we used was the 100 micron. We have in the past, as you say, seen some problems but we corrected them by washing the aperture daily with Chlorox. I know this will change. We do happen to have the mean cell volume computer, and this picks up very nicely that your MCV's are beginning to increase when the aperture is actually decreasing by a buildup of protein. And so we clean these daily and you can't see a buildup that way. We use the standardization method supplied by Coulter now, using their 4C standard, which has taken out a considerable amount of guess work. We were in the dark when we first started about three or four years ago. We just had to do duplicate samples to see if it counted the same at the end of the day as at the start.

DR. MAC FARLAND: I would like to make a couple of comments and ask a question. I've been using macaques in long term inhalation studies for some time and I would certainly support the remarks Dr. Hall has made. I'm thinking particularly here of the difficulties of working with Rhesus monkeys, the problem of the lung mite infestation and their very high susceptibility to tuberculosis. It is very hard to think of a justification for using this particular species in inhalation studies if you are concerned with measuring toxicological parameters or anything that has to do with the physiology or pathology of the lung. We used M. Iris, the cynomologous, and if you're going to use a macaque I think this is a very good one myself. They're highly resistant to tuberculosis, they do suffer from lung mite infestations, but our experience has been that this is very markedly less than you normally encounter in the Rhesus. They also have, for studies of pulmonary function, a very nice practical advantage, and that is they have an elongated dog-like snout and this is very handy when you're fitting them with face masks. This is hard to do in the Rhesus with his flat face. The second comment has to do with the difficulty of getting perhaps true hematological parameters, particularly the electrolytes, the blood gases, and the pH in animals you have just captured and are excited. Our experience has been that the only way you can get a value that you feel is not artificially elevated or depressed and also, where you want to have a standard deviation which isn't as big as the absolute value of the parameter itself is to use Sernylan, and this brings everything into line beautifully. Finally, the question. Your statistical summary tables, these are for both sexes combined?

DR. HALL: Yes, right, but we do have data according to sex if anyone would be interested. When we had the data arrayed it was arrayed by individual sexes and for both. We have used other methods for determining BUN's and proteins. We used to use a salting-out biuret method, for example, and those comparisons didn't seem relevant here actually so this was more a summary of both sexes so I could sort of just summarize clinical information. But there are, by inspection, definite sex differences particularly hematocrits in females and male Rhesus, and there is a difference in standard deviation which one might expect with the menstrual cycles.

DR. BOYD (Aerospace Medical Research Laboratory): One quick question on your white counts that you attribute to lung mites. Do you think that if you selected animals either in the level of your means for white counts or below that you would be avoiding a very serious lung mite problem in the particular group?

DR. HALL: Well, in conjunction with radiographs--if you have a good clean radiograph field and the animal, some of the psychologists have laboratory reared animals that they have separate caged, have never been caged double--they have clear lung mite fields and definitely in these animals you can expect the lower mean white count. The differential count will be usually 60% lymphocytes, 30% mature neutrophils, and maybe one eosinophil. Now this is assuming that there are no parasites. But in our adult males that have been captured and been in our colony for three or four years and have a loaded field of lung mites, definitely these animals tend to have a more deviating white count. It may be low sometimes, but the next time it's high because they're continually having chronic bacterial infections in the lung.

FREEZE-CLEAVED LUNG. A PRELIMINARY REPORT ON  
THE ULTRASTRUCTURE OF THE ALVEOLAR SAC IN NORMAL MOUSE\*

Ronald S. Weinstein, M. D.\*\*

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

James C. Hogg, M. D., Ph. D.

Gerald Nash, M. D.  
and  
N. Scott McNutt, M. D.

Massachusetts General Hospital  
Boston, Massachusetts

### INTRODUCTION

It is widely held that the alveolar sac of mammalian lung is lined by a layer of extracellular material that is capable of lowering the tension at the air-surface interphase. The morphology of such a sac lining layer has been difficult to demonstrate with conventional thin section electron microscopy because of certain technical limitations imposed by thin section techniques (Gil and Weibel, 1969; Dermer, 1969). In this study we have applied an alternative electron microscopy preparative technique called freeze-cleaving (Weinstein and Someda, 1967) or freeze-etching (Moor, 1966) to the problem of demonstrating extracellular alveolar sac lining layers. This technique permits examination of the sac wall without tissue fixation, dehydration or embedding in plastic. Further, with freeze-cleaving large areas of the topography of the alveolar sac wall are seen in three-dimensional relief. In this preliminary report we describe some observations on selected membrane systems in freeze-cleaved mouse lung. Some aspects of this work are reported elsewhere by Nash and Weinstein (1968).

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\*\* This work was carried on at the Department of Pathology and Mixter Laboratory for Electron Microscopy, Neurosurgical Service, Massachusetts General Hospital, Boston, Massachusetts and the Department of Pathology, Harvard Medical School, Boston, Massachusetts.

## METHODS

Adult Charles River mice weighing 12 to 17 grams were fed ad lib and sacrificed by a heavy blow on the head. Animals were separated into three groups. Two groups were used for freeze-cleaving and one for thin sectioning. Group I animals were sacrificed and their lungs gently sliced into 1 mm<sup>3</sup> blocks. The tissue was fixed by immersion for 15 to 30 minutes in 2% glutaraldehyde buffered with sodium cacodylate, pH 7.4. Blocks were bathed at 0 C in a cryoprotective solution (1.0M sucrose in Tyrodes solution) for 30 minutes or overnight (figure 1, left). With this protocol, residual gas in alveolar sacs is replaced by the cryoprotective solution. Tissue blocks were mounted on small freezing cartridges and rapidly frozen in liquid Freon 22 cooled to approximately -150 C.

Group II animals were sacrificed and their lungs were rapidly removed and similarly cut into small blocks. The blocks were mounted directly on brass freezing cartridges and frozen immediately in Freon 22 at approximately -150 C without exposure to either cryoprotective agents or chemical fixatives. The average elapsed time from sacrifice of the Group II animals to freezing of lung blocks was less than one minute. In these specimens, the alveoli contained air at the time of quenching and liquid nitrogen at the time of cleaving as described in figure 1, right.

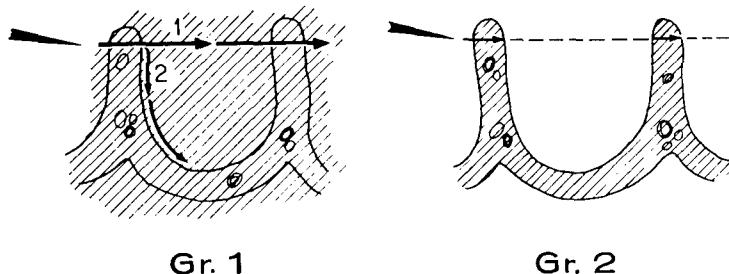


Figure 1. CRYOPROTECTION FOR FREEZE-CLEAVING. Schematic representation of the alveolar sacs at the time of cleavage. Sacs of Group I animals (Gr. 1) contain frozen sucrose solution. The cleavage may pass directly into the sucrose solution (Path #1) or be deviated along a membranous structure at the sac wall (Path #2). Air sacs of Group II animals (Gr. 2) contain liquid nitrogen. Cleavages tend to pass directly across alveolar sacs. After removal of the nitrogen in vacuo, the sac wall is accessible for direct replication.

The general protocol for freeze-cleave and etch techniques is outlined in figure 2. In this study, frozen specimens from Group I and Group II animals were cleaved with a precooled razor blade under liquid nitrogen at -196 C (Bullivant and Ames, 1966) and heat etched in a Type II MGH freeze-cleave device (Weinstein and McNutt, 1970). For freeze-cleave, non-etch experiments, the specimen was maintained at a temperature below -175 C throughout the replication procedure. In heat-etch experiments, the maximum temperature was -105 C and replicas were cast at  $-110 \pm 2$  C. Carbon-platinum replicas of fracture faces were cast in an unmodified Kinney evaporator (Model PW 400) at a vacuum of  $< 1 \times 10^{-5}$  mm Hg. Platinum shadowing was at a 45 incident angle to the plane of the specimen holder. After replication, the specimen was thawed and the tissue was washed away from the replica by successive washes in household bleach (Chlorox®) and distilled water. Replicas were retrieved on Formvar coated copper grids and photographed in a Siemens Elmiskop I transmission electron microscope.

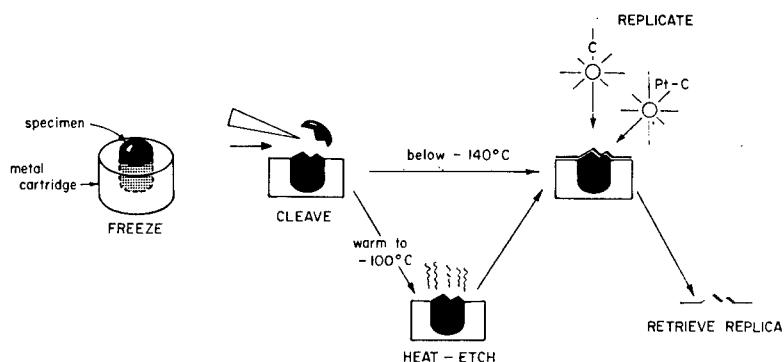


Figure 2. FREEZE-CLEAVE PROTOCOL. Tissue is frozen in a metal cartridge and cleaved at low temperature with a precooled razor blade. The newly generated fracture face is replicated at low temperature by evaporating platinum and carbon onto it in a vacuum system. The condensing platinum-carbon mixture forms a coherent replica of the topography of the fracture face. After thawing the specimen, the replica is retrieved and examined in a transmission electron microscope. An optional step called "heat-etching" can be added to the protocol to demonstrate some structures that are not visualized with cleaving alone.

Thin sections - Tissue from Group III animals was used for thin sections. One mm<sup>3</sup> blocks of lung were fixed by immersion for one hour in a cold paraformaldehyde/glutaraldehyde/picric acid mixture buffered with 0.1M cacodylate at pH 7.2 to 7.4. The blocks were post-fixed with cacodylate buffered 2% osmic acid, stained en block with 1% uranyl acetate in veronal-acetate buffer and embedded in Epon 812. Thin sections were cut on diamond knives, stained with uranyl acetate and lead citrate, and photographed in a Siemens Elmiskop I electron microscope.

## RESULTS

Plasma membrane ultrastructure - Cleavage planes passing through frozen tissues are frequently deviated along membranes and reveal large areas of membranes in three-dimensional relief at the fracture face generated during cleavage. Aspects of membranes exposed in this manner are replicated and the replicas are examined electron microscopically (figure 3).

The aspect of plasma membranes demonstrated by freeze-cleaving has been controversial. According to one view, originally proposed by Muhlethaler and his associates (Muhlethaler, Moor and Szarkowsky, 1965), two surfaces of membranes are revealed by cleaving. They believed that the surfaces represent the true surfaces of the membrane. Early evidence appeared to support this suggestion (see reviews by Moor, 1966; Koehler, 1968; and Weinstein, 1969). However, more recent evidence favors an alternative interpretation proposed by Branton in 1966. Branton agreed that cleavages of membranes reveal two distinctive surfaces. He suggested that frozen membranes are split into two lamellae and it is the new surfaces originating from the membrane's interior that are revealed by cleaving. According to this interpretation, a novel view of the internal structure of membranes would be achieved with the freeze-cleave technique. Branton suggested that true outer surfaces of membranes are revealed exclusively by heat-etching (Branton, 1966; Pinto da Silva and Branton, 1970). Although the Muhlethaler hypothesis is still favored by some investigators, we find that our results with lung are best explained by the Branton interpretation.

The two faces of plasma membranes that are exposed by freeze-cleaving are called the "A-Face" and the "B-Face". The orientation of a fracture face of a membrane can be determined by examining replicas. By convention, the fracture face of a plasma membrane oriented toward the cell exterior is labeled "A-Face" and the face oriented toward the cell cytoplasm is called its "B-Face" (Branton, 1969; McNutt and Weinstein, 1970; Weinstein and McNutt, 1970). A single cleave along the interior of a plasma membrane generates one A-Face and one B-Face and in a given area of replica either an A-Face or a B-Face may be seen for a single membrane.

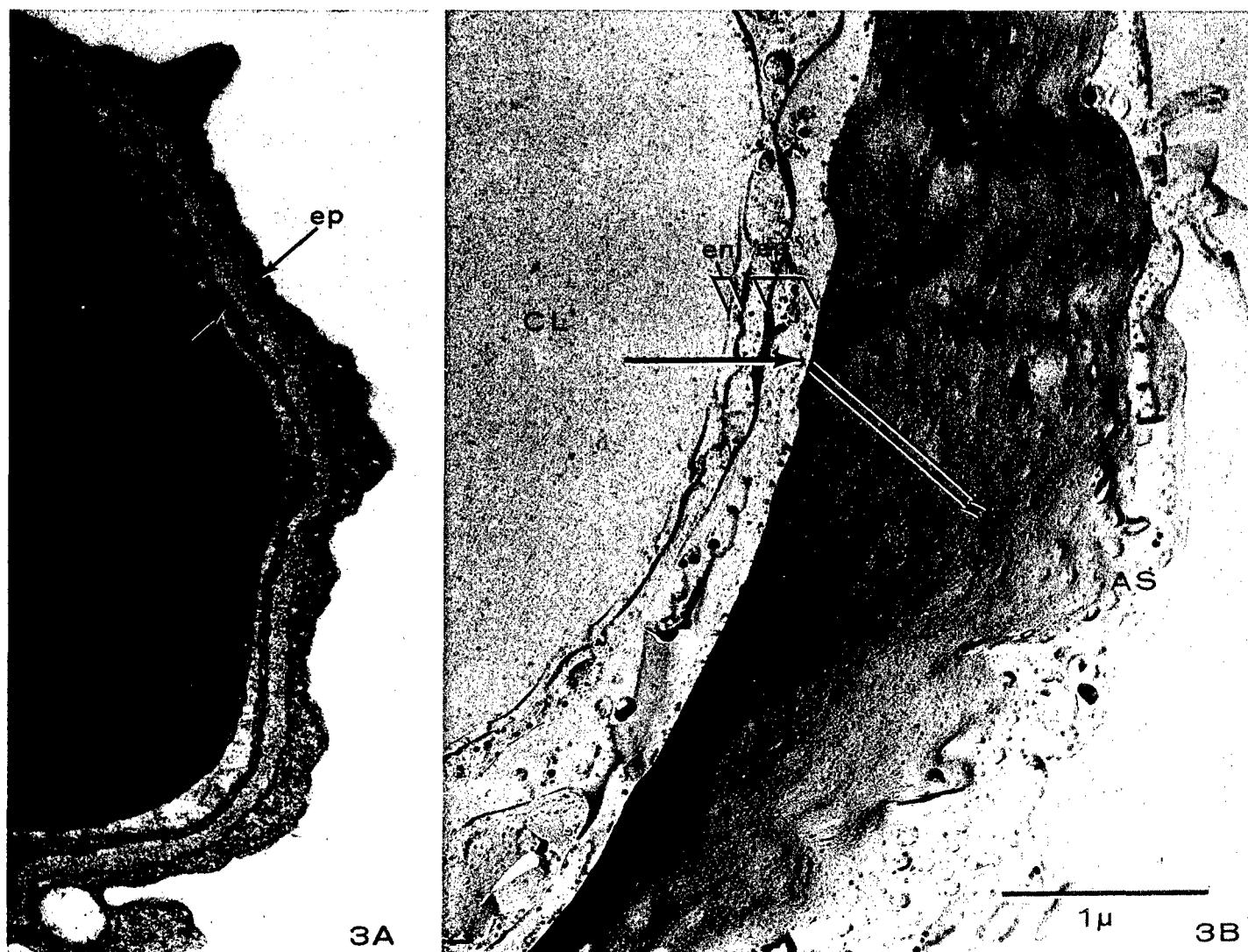


Figure 3A & 3B. ELECTRON MICROSCOPY OF THIN SECTIONED (3A) AND FREEZE-CLEAVED (3B) MOUSE LUNG. A thin section (3A) shows essentially a two-dimensional representation of plasma membranes. In 3A, the epithelial (ep) and endothelial (en) cells are attenuated. A red blood cell (RBC) is in the capillary lumen. The alveolar sac is to the right. The hypophase and surface film (layer) are inapparent in this preparation. 3B is a freeze-cleave preparation. The cleavage (arrow) has passed through the capillary lumen (CL), endothelial cell (en) and alveolar epithelial cell (ep) and has been deviated for a long distance (double arrow) along the epithelial cell membrane revealing a large area of the plasma membrane in three-dimensional relief. The cleavage finally enters the alveolar sac (AS). X 31,000

The A-Face and B-Face of all plasma membranes in lung, including plasma membranes of epithelial cells, endothelial cells and interstitial cells, are studded with a sparse population of small particles which measure in replicas from approximately 60 to 100 $\text{\AA}$  in diameter as measured in the plane of the membrane (figure 4B and 5C). These particles are often more plentiful on an A-Face than a B-Face for a given cell type. The particles on plasma membrane A-Face and B-Face for cells of the alveolus are morphologically indistinguishable from the membrane-associated particles on natural membranes in a variety of other tissues with this technique. Such particles are absent from artificial lipid membranes (figure 4A). A discussion of the chemical identity and functional role of the A-Face and B-Face particles is beyond the scope of this presentation. In the context of this paper, we are mainly interested in using the particles as ultrastructural markers for plasma membranes.

Surface film (alveolar sac lining membrane) - The cleavage plane, after passing through or along an epithelial plasma membrane in lung enters the alveolar sac where a surface film or alveolar sac lining "membrane" is encountered (figure 5A & C). The surface film in alveolar sacs infused with sucrose solution (Group I animals) usually appears as a single sheet, is extremely thin (<50 $\text{\AA}$ ), and is non-etchable. Such sheets are totally devoid of membrane-associated particles that are characteristic of cell membranes in general. The surface film is focally wrinkled but generally follows the contour of the alveolar sac. In some areas the surface film appears to be in contact with Type I alveolar cell membranes while in other areas the film can be separated from the alveolar cell membrane by an etchable fluid phase measuring up to several microns or more in thickness. Whereas the surface film can be in close apposition to Type I alveolar cells, it only approaches Type II cells at the tips of their apical microvilli. The film is a flat sheet lying over microvilli rather than forming a cast of the complicated apical surface topography of the Type II cells.

The microvilli of Type II alveolar cells in Group I animals are bathed in etchable fluid which contains ice crystals of comparable size to those of the sucrose solution filling the alveolar air space. Occasionally, round to oval fragments of smooth particle-free non-etchable membrane extend from the apical surface of Type II cells across the fluid filled compartment (hypophase) to the sheet-like alveolar lining layer. These fragments indent focally the surface film and appear to represent the incorporation of membranous material into the surface film. In Group I animals areas of surface film up to several thousand square microns have been examined en face without demonstrating evidence of the suture lines between the underlying epithelial cells. This suggests that the surface film is continuous over sutures.

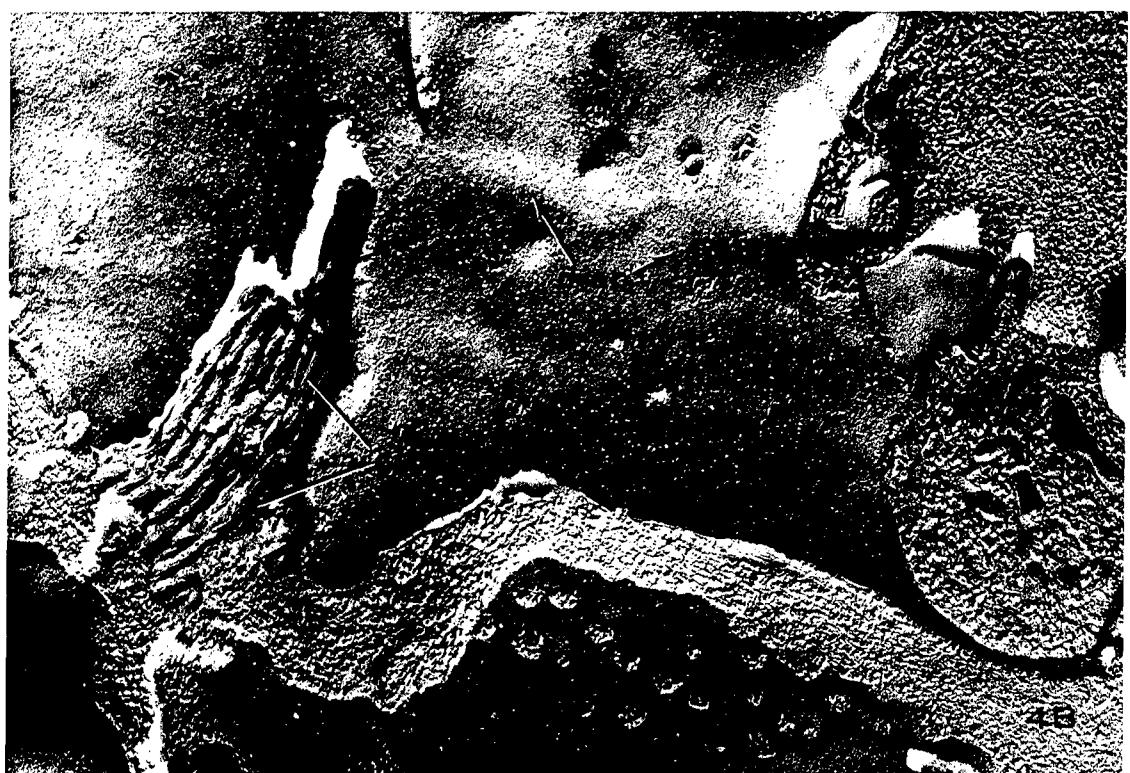
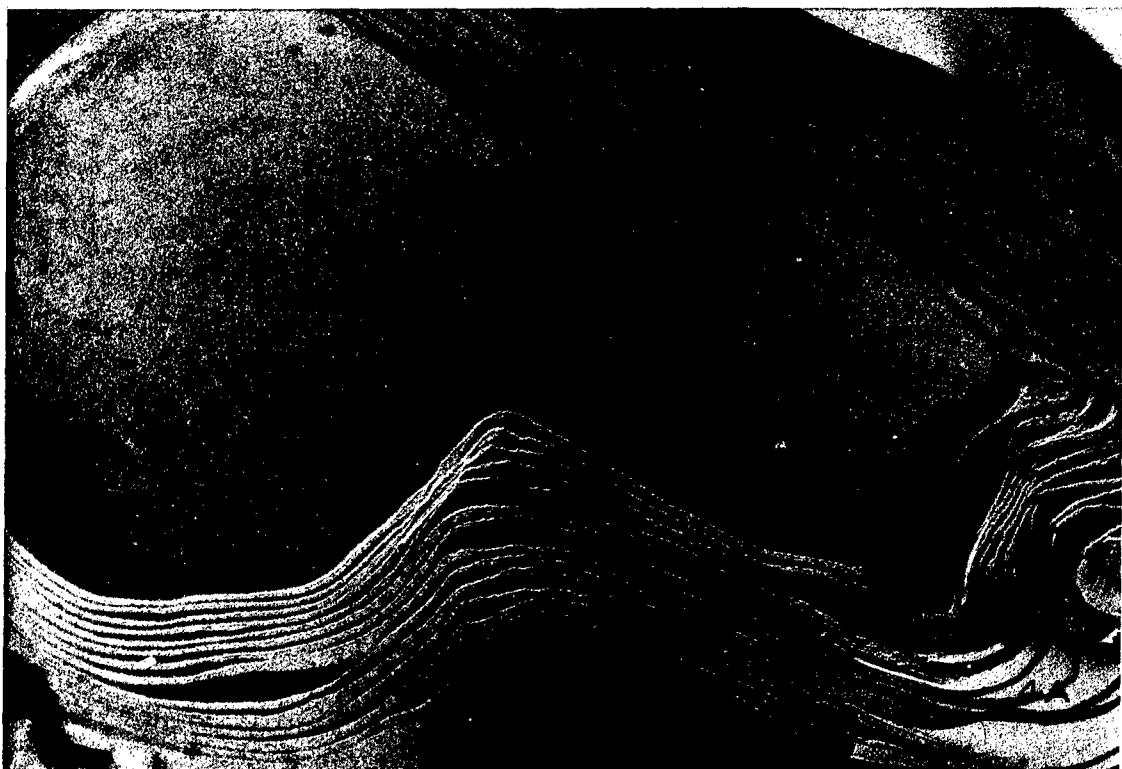


Figure 4A & 4B. COMPARISON OF FREEZE-CLEAVED ARTIFICIAL LIPID MEMBRANES AND PLASMA MEMBRANES. 4A - Replica cast of a fracture face produced by an eccentric cleavage through artificial membranes produced in an aqueous solution of purified brain phospholipid. The membranes are smooth to finely granular and are particle-free. 4B - Replica of freeze-cleaved lung. Plasma membranes are studded with 60 to 100 $\text{\AA}$  membrane-associated particles (map). A bundle of collagen is in the interstitium. X 50,000



Figure 5A-D. FREEZE-CLEAVED SURFACE LAYER (FILM). Figure 5A, B and C are from Group I animals. 5A - The cleavage has passed through the etchable "hypophase" (top) and has been deviated along the alveolar sac surface layer (surface film) which is non-etchable. The film is focally breaking down into myelin figures (mf). x 38,000. 5B - Eccentric cleavage through a myelin figure generated from the surface film. x 64,000. 5C - The cleavage has passed along the alveolar epithelial cell A-Face (ep-A), through the hypophase (X) and along the alveolar sac surface film (layer). The A-Face is studded with membrane-associated particles while the surface film is particle-free. Several fragments (arrows) of surface film have been stranded over the A-Face by the cleavage. x 54,000. 5D - Replica at the base of an alveolar sac in a Group II animal. x 76,000

To view the true outer surface of the alveolar lining membrane in addition to the novel faces generated by cleaving as in the Group I animals, lung blocks from Group II animals were freeze-cleaved. In these experiments cleavages pass through frozen alveolar septa directly into air spaces (figure 1, right). Since the cleaving is done in liquid nitrogen (-196 C), the air sacs are filled with liquid nitrogen at the time of cleavage. The nitrogen in the air sacs freezes when the specimen temperature is depressed from nitrogen evaporation in vacuo and the solid nitrogen then sublimes away in vacuo. With removal of nitrogen from an air sac the tissue-gas interface of the alveolus is exposed for replication. Presumably this face is the same as the outer surface of the alveolar sac lining membrane (surface film). Replicas of the surface exposed in this manner show it to be gently convoluted and non-etchable (figure 5D and figure 6). At high magnification it appears smooth to finely granular. The surface is continuous and, although ice crystals either in the underlying cells or in the hypophase occasionally distort it (figure 6), contours of suture lines between underlying cells and of microvilli were not visualized at this surface.

Lamellar bodies - Cleavages through Type II alveolar cells reveal various views of lamellar bodies (figure 7A, B & C). A cleavage may pass: (1) exclusively along the lamellar bodies limiting membrane without revealing lamellae; (2) may expose areas of both limiting membrane and the lamellae; or (3) pass directly through the limiting membrane revealing fracture faces of lamellae exclusively. The limiting membrane of lamellar bodies (figure 7C) resembles cytomembranes and plasma membranes in that it is studded with the distinctive 60 to 100 $\text{\AA}$  membrane-associated particles previously described. In contrast, the fracture faces of lamellae of these bodies are smooth and topographically indistinguishable from the fracture faces of the surface film (Group I animals). Individual lamellae are estimated to be approximately  $45\text{\AA} \pm 5\text{\AA}$  in thickness; are flat or gently curved and are usually tightly packed (figure 7B & C). The appearance of freeze-cleaved lamellar bodies is consistent with their appearance in thin sections (figure 8), although it is noteworthy that they appear less distorted in the freeze-cleave preparations.

## DISCUSSION

Our observations on freeze-cleaved mouse lung confirm and extend the results reported by several other groups using conventional thin section electron microscopy.

Overlying the alveolar cell membrane and facing into the alveolar sac is a highly hydrated layer of variable thickness that has been referred to as the "hypophase" or "base layer" (Gil and Weibel, 1969; Scarpelli, 1968). As seen in sections and in replicas of freeze-cleaved material, this layer fills in the crevices between cells, the irregularities at the cell surface and surround the numerous microvilli of the Type II cells. The layer varies in thickness from under 100 $\text{\AA}$ , where it appears to be in focal intimate contact with Type I cells in freeze-cleave preparations, to over several microns. The hypophase is seen regularly in thin sections only in material prepared with fixation techniques specifically devised to preserve it (Gil and Weibel, 1969; Deamer, 1969). The hydrated layer is seen in freeze-cleave specimens as an etchable layer of variable thickness that closely corresponds in its distribution to that described in thin sections by others.

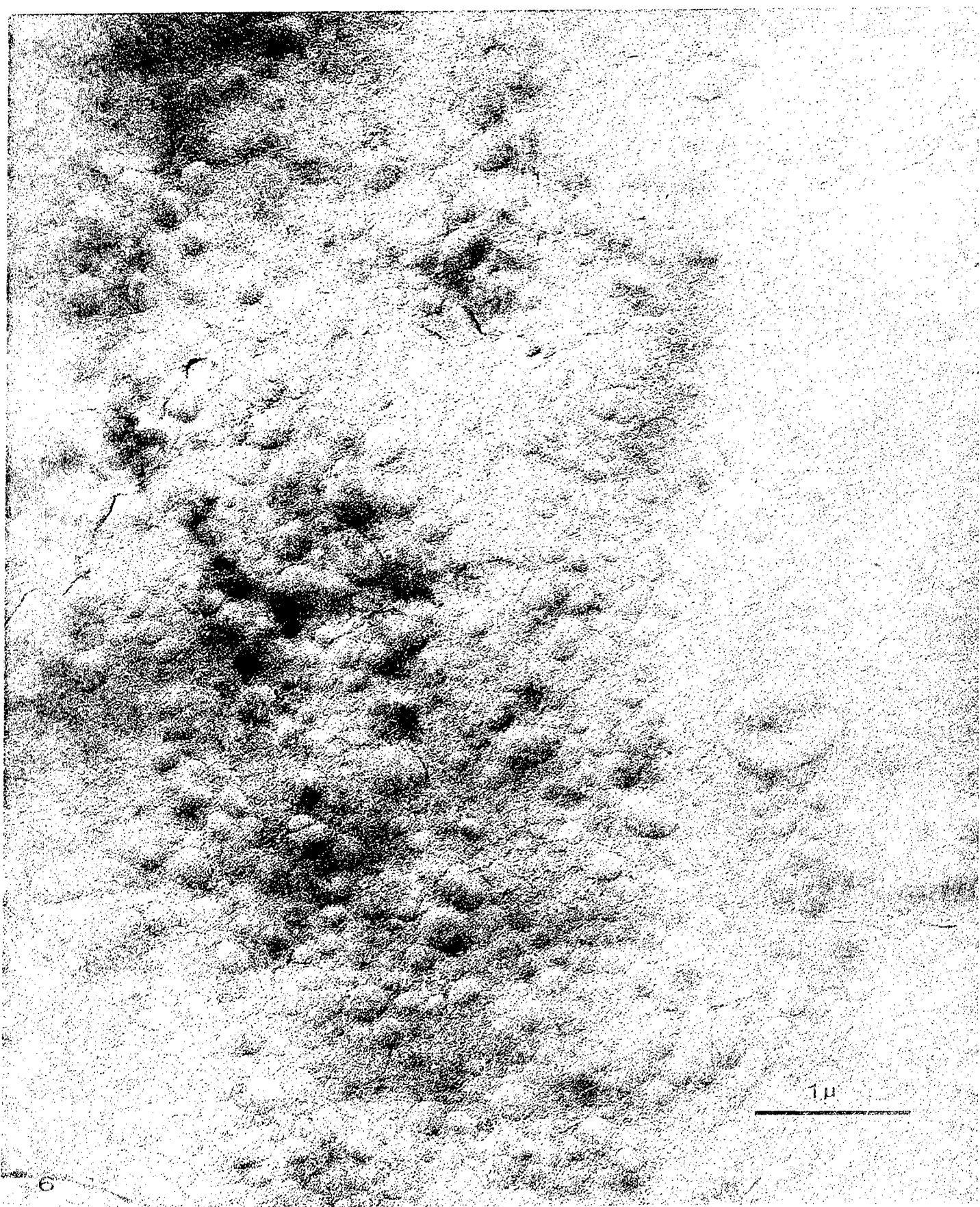


Figure 6. REPLICA OF MOUSE ALVEOLAR SAC IN A GROUP II ANIMAL. X 30,000

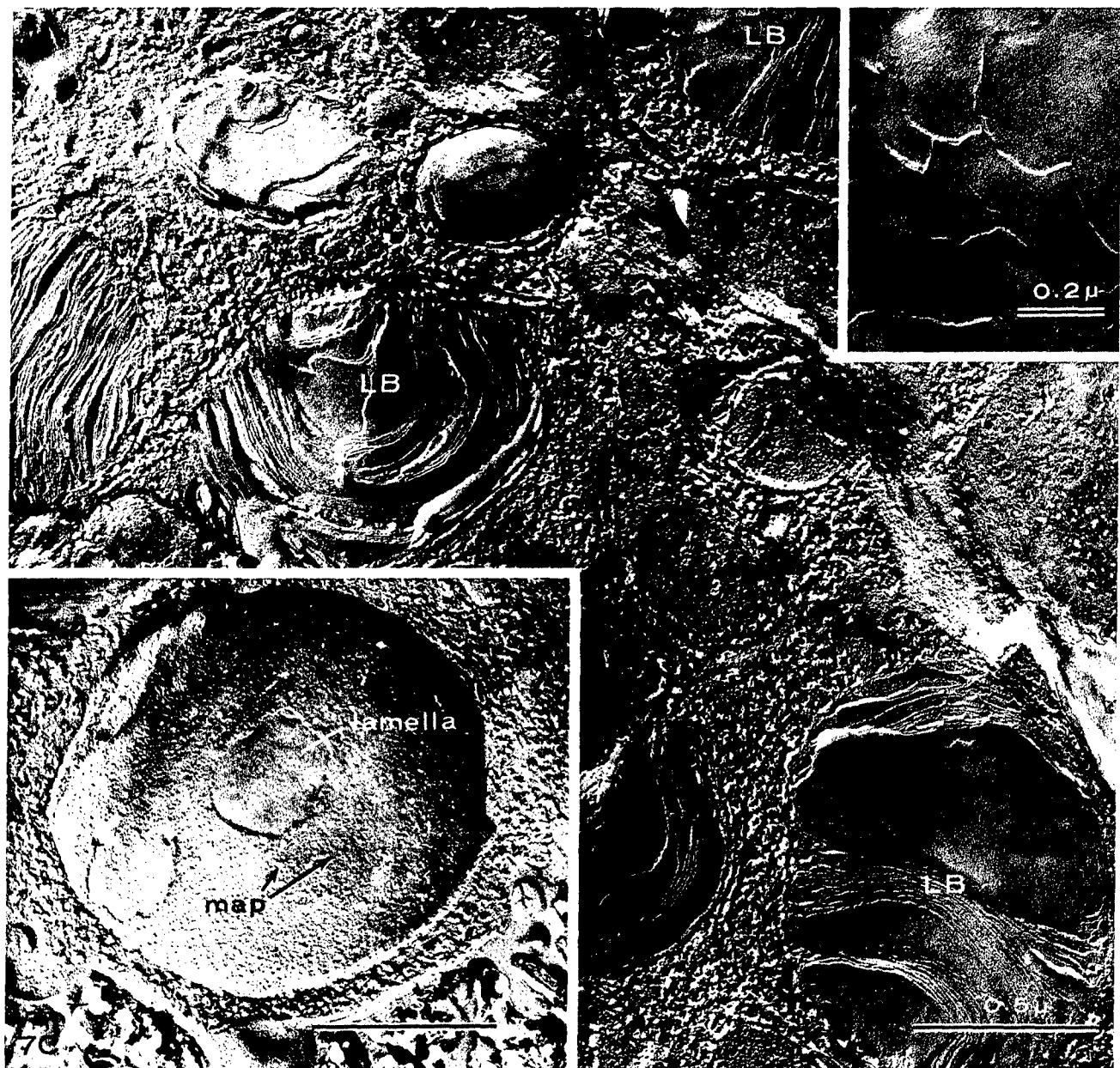


Figure 7A-C. FREEZE-CLEAVED LAMELLAR BODIES IN MOUSE LUNG.  
7A - Eccentric cleavages through the packed lamellae in lamellar bodies (LB). X 56,000. 7B - High magnification of lamellae showing their smooth fracture faces. X 65,000. 7C - The limiting membrane of a lamellar body is studded with membrane-associated particles (map). A lamella, left behind by the cleavage, has a smooth fracture face. X 56,000

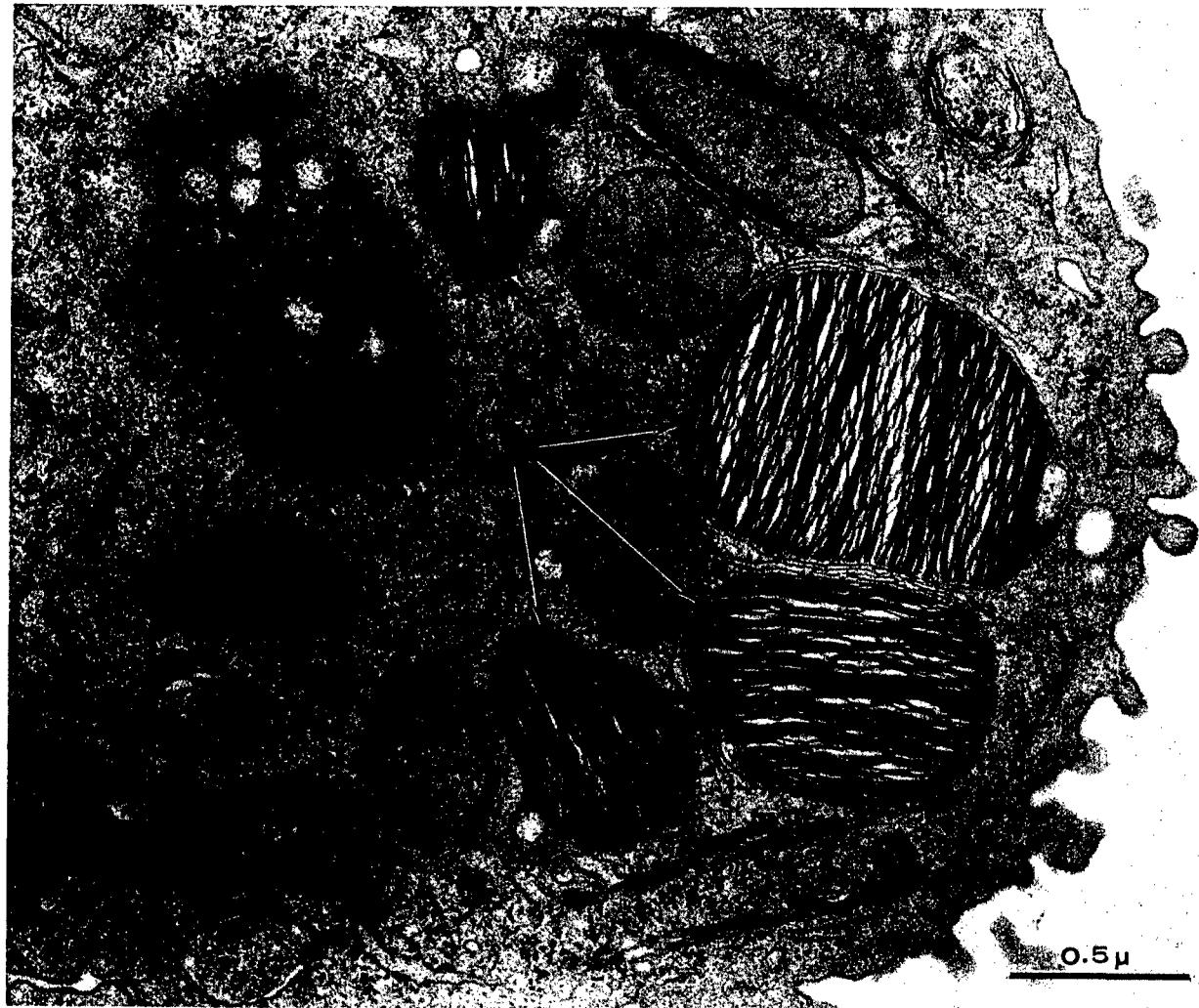


Figure 8. THIN-SECTIONED LAMELLAR BODIES. Lamellae of lamellar bodies (LB) are osmiophilic and appear less tightly packed than in freeze-cleave preparations (compare with figure 7). Mitochondria are prominent in the apical cytoplasm. X 50,000

In thin sections, the hypophase appears to be covered by an extremely thin layer of osmophilic material of molecular dimensions (Gil and Weibel, 1969; Petrik and Riedel, 1969; Dermer, 1969; Lambson and Cohn, 1968) which is often difficult to demonstrate with standard thin section techniques. Although it has been suggested that the osmophilia of the surface film indicates a lipid composition, the specificity of osmophilia is debatable (Korn, 1967).

The existence of a cleavage plane within the surface film gives direct evidence that this structure represents a membranous physical barrier rather than simply an interface between two compartments that non-specifically accumulates heavy metal electron stains. It also lends indirect support to the notion that the surface film is lipid since lipid membranes readily deviate cleavages (Deamer and Branton, 1967) and have smooth, particle-free fracture faces (Weinstein, Ivanetich and Nash, 1968; Deamer, Leonard, Tardieu and Branton, 1970).

The existence of a cleavage plane along the surface film suggests that the film exists as a bilayer although our data are by no means conclusive on this point. Single membrane thicknesses are notoriously difficult to measure from replicas and the apparent thickness of the alveolar sac lining membrane in our material is probably consistent with either a monolayer or bilayer configuration. Double replica experiments (Steere and Moseley, 1969) would probably shed further light on this point. Such experiments would establish if two non-etchable faces are generated from the surface film by cleaving. However, it would still be difficult to interpret the results in terms of the in vivo configuration of the surface film since the possibility that incorporation of lipid from the hypophase into the surface film with a conversion from a monolayer to bilayer during handling cannot be entirely eliminated in experiments involving the introduction of a cryoprotective agent into the alveolar sac.

Although the morphologic appearance of the fracture face topography does not yet allow conclusions to be drawn about the chemistry of membrane components, the membrane-associated particles serve as a useful morphologic marker. Of the many membrane systems thus far described, only nerve myelin (Branton, 1967) and artificial lipid membranes (Deamer and Branton, 1967; Weinstein, Ivanetich and Nash, 1968; Deamer, Leonard, Tardieu and Branton, 1970) lack these membrane-associated particles. The demonstration in this study that the particles are also absent in the stacked lamellae in lamellar bodies of the Type II alveolar cells and the lining film of the alveolar sac is consistent with the suggestion that these membranes have a common identity.

Our results are in essential agreement with the conclusion of Gil and Weibel (1969) that the hypophase separating the alveolar surface film from alveolar epithelial cell membrane is of variable thickness. There are theoretical reasons to support the concept of local variations in the thickness of the hypophase. Clemments and Tierney (1965) have suggested a physical-chemical interrelationship between the surfactant (surface) film and hypophase. They suggest that the surface tension of the film may be capable of determining the composition of the hypophase through its effect on fluid balance in the alveolar wall, while the hypophase is capable of altering the surface

tension of the surface film through changes in its composition. If such an interrelationship exists it is unlikely that it would be at exactly the same stage over the entire lung surface so that one might expect both local and regional differences in hypophase thickness. Although this study does not provide direct evidence to support such interrelationships, the freeze-cleave technique demonstrates interesting morphologic features of the alveolar wall and may prove of value in the study of the complex interaction between the surface film, the hypophase and underlying cells.

#### NOTE OF ADDED PROOF

After the completion of this study, a report by Meyer et al on freeze-cleaved lung came to our attention (H. W. Meyer, H. Winkelmann and W. Kühne, Elektronenmikroskopische Untersuchungen an der Saugetierlunge mit Hilfe der Gefrieratzttechnik. I. Die Darstellung der Alveolenoberfläche. Exp. Path., 3, 138, 1969). Our results are in essential agreement with those described in their report.

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## DISCUSSION

DR. TOWNSEND (University of Texas Medical School): Needless to say this is, to me at least, a very exciting area. Seeing things in the alveolus that I had never seen before, and really visualizing the structure there which is going to be so vital in understanding some of the changes that go on.

COLONEL ROBINSON (Armed Forces Institute of Pathology): I wanted to ask Major Weinstein if he's visualized some of these surface active particles penetrating the type I cells as I recently saw suggested in a journal? The so-called surfactant apparently penetrates the type I cells and is excreted in that direction.

MAJOR WEINSTEIN (Aerospace Medical Research Laboratory): It is difficult to know what direction things move on the basis of static micrographs. We have seen, in freeze-cleave preparations, small vesicles associated with the type I epithelial cell membrane both at the tissue front and at the air sac. These vesicles appear to represent pinocytotic vesicles and are 600 to 700 $\text{\AA}$  in diameter. They could be transporting material either into the cells or out of the cells. The vesicles opening into the air sac could conceivably contain fragments of surface active material although we have obtained no evidence for this from our freeze-cleave preparations. I would emphasize, however, that we have not specifically looked for smooth membranous fragments in these vesicles.

DR. DU BOIS (University of Pennsylvania): It is very exciting to see these layers in the lung. They have been seen in material washed out of the lung and then deposited on a grid and again you look at the surface of the layer. The question I had was whether this layer is one layer that you see on the surface or whether you slice through many layers of lamellae in the alveolus.

MAJOR WEINSTEIN: That is a very pertinent question. We have seen (G. Nash and R.S. Weinstein, Amer. J. Path., 52: 18A, 1968) that in glycerol infused preparations, some areas of the alveolar sac can appear to have several layers. However, we suspect that this appearance might be artifactual. I believe that a judgement on the multiplicity of layers will ultimately have to be made from material which has not been exposed to a cryoprotective agent (e.g. glycerol) since it is likely that some of the surface active

material would migrate and perhaps stack during the interval when the cryoprotective agent is infiltrating the tissue. A number of authors have published micrographs of thin sections showing stacked layers of membranous material on the alveolar wall in some areas and denuded areas of sac wall in other parts of the same sections. This appearance may suggest that portions of the surface active layer may migrate during preparation procedures. Extremely rapid freezing of minimally handled lung tissue may provide us with preparations which will yield a more conclusive answer to your question. We plan to try that approach.

SESSION IV

ATMOSPHERIC CONTAMINANTS

Chairman

Dr. Keith H. Jacobson  
Laboratory of Environmental  
Medicine  
Tulane University School of Medicine  
New Orleans, Louisiana

## THE AUTOMATED GAS CHROMATOGRAPH AS AN AIR POLLUTANT MONITOR

Robert K. Stevens

National Air Pollution Control Administration  
Raleigh, North Carolina

### INTRODUCTION

Gas chromatography has been used extensively over the past 10 years to measure atmospheric concentrations of a variety of air pollutants. Altshuller et al (1966) and Kippe et al (1967) are among some of the early investigators who have used this powerful analytical tool as an aid to determining the composition of polluted atmospheres.

However, only recently has the gas chromatograph been thought of as an analytical system which could serve faithfully as a routine air pollution monitor. Stevens and O'Keeffe (1970) and Stevens, Ortman and O'Keeffe (1968) have recently demonstrated that an automated gas chromatograph could be used to measure ambient as well as source concentrations of carbon monoxide, methane, sulfur dioxide and hydrogen sulfide.

This paper will present details of these analytical developments and discuss their role in future monitoring programs.

### METHODS

#### A. Description of Prototype Carbon Monoxide and Methane Analyzer

The chromatographic analyzer shown in figure 1 was developed to measure ambient concentrations of carbon monoxide and methane. The system can also measure total hydrocarbons by incorporating a second valve to introduce a known volume of air directly into the flame detector, bypassing the columns and catalytic reactor.

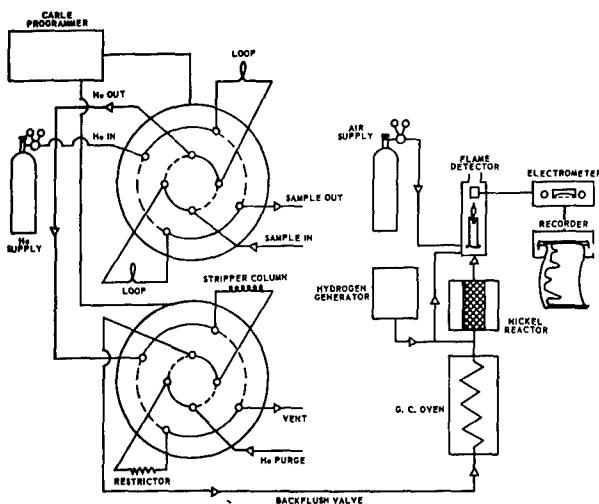


Figure 1. AUTOMATED METHANE, CARBON MONOXIDE CHROMATOGRAPH

The analysis of air samples is performed by introducing a known volume of air into a chromatographic column where the methane and carbon monoxide are separated. The methane passes through a catalytic converter unchanged and into the flame ionization detector. The methane component is followed by carbon monoxide which is converted to methane as it elutes from the column and passes through the converter to the flame.

The analytical system (figure 1) consists of the following:

Micro-volume sampling valve with two 15-ml sample loops

(Carle Valve Co. No. 2014 with No. 2050 automatic actuator).

Column switching valve (Carle Valve Co. No. 2014 with

No. 2050 automatic actuator).

Time sequence programmer (Carle Micro Volume Valve Programmer PH 2100).

Stripper column: a 12-inch length of 1/4-inch-OD stainless steel tube packed with 5 inches of 10% Carbowax-400 on 60/80 mesh Chromosorb W HP, 5 inches of 60/80 mesh silica gel, and 2 inches of Mallcosorb (Mallinckrodt).

Perkin-Elmer 800 Gas Chromatograph.

Gas Chromatograph column: a 12-foot length of 1/4-inch-OD stainless steel tube packed with 5A molecular sieve, 60/80 mesh.

Catalytic reactor: a 6-inch length of 1/4-inch-OD stainless steel tube packed with 10% nickel on 42/60 mesh C-22 firebrick.

Flame ionization detector (Micro-Tek Instruments, Austin, Texas).

Victoreen Electrometer Model No. 4010-3.

Recorder: Honeywell Model 15, minus 0.05 to plus 1.05 mv.

Pump Neptune Dyna-Pump Model 2.

The operating conditions were as follows:

Temperatures, °C

Stripper Column:	25 ± 5
Molecular sieve column:	115
Detector:	150
Reactor:	360

Gas Flows, ml per minute

Helium Carrier:	200
Hydrogen:	To reactor: 30 To flame detector: 60
Air:	To flame detector: 400

### PREPARATION OF CATALYTIC SURFACE FOR CONVERSION OF CARBON MONOXIDE TO METHANE

To 10 grams of 42/60 mesh C-22 firebrick (Coast Engineering Laboratory, Hermosa Beach, California) were added 24 ml of an aqueous solution containing 50 grams of  $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ . The mixture was slowly dried in a fluidizer (Applied Science, State College, Pennsylvania) at 110 C while being purged with a stream of dry nitrogen flowing at 300 ml per minute. Lumps formed during the drying process were gently broken, and the material was packed in a 6-inch-long, 1/4-inch-OD stainless steel tube. The tube was heated to 500 C for one hour while being purged with oxygen at 100 ml per minute. The reactor was cooled, then installed downstream from the molecular sieve column (figure 1) and slowly heated to 360 C while being purged with a mixture of 200 ml per minute helium and 30 ml per minute of hydrogen for two hours. For optimum results, the reactor was maintained at 360 C with the helium-hydrogen gas mixture (at the 200/30 ratio) flowing through it.

### Sampling and Analysis

Procedure 1: Sample air is pushed through the sample loop at a rate of 100 ml per minute with a diaphragm pump positioned between the sample source and the sample loop. A sample is injected into the analyzer every 10 minutes. The sample then flows through the loop into the stripper column before entering the molecular sieve column. After 30 seconds the backflush valve actuates, reversing the carrier flow in the stripper column to vent while maintaining the flow through the molecular sieve column. Oxygen and nitrogen are eluted first from the molecular sieve column into the reactor and flame detector causing baseline disturbance of signal. Following the oxygen and nitrogen is the methane, which in turn is followed by the carbon monoxide. The catalytic reactor quantitatively converts the carbon monoxide to methane before it is detected by the flame ionization detector.

Procedure 2: Instead of the sample being pumped directly into the sample loop from the air source, the sample is first pulled through an integrating vessel. The volume of the vessel and the flow rate through the vessel are selected so that the sample delivered to the gas chromatographic system represents the concentration averaged over the sample residence time in the vessel, which in turn is selected to correspond to the sampling interval. This procedure approximates the average concentrations of methane and carbon monoxide that prevail in the atmosphere between introductions of sample to the chromatograph.

Atmospheric Air Analysis for Carbon Monoxide and Methane: This analytical system has been used almost continuously for two years to measure ambient concentrations of carbon monoxide and methane. Although methane is not considered an important air pollutant, it can provide a rough though useful index of the atmospheric hydrocarbons capable of participating in formation of photochemical smog (total hydrocarbon concentration minus methane concentration approximates concentrations of reactive hydrocarbons).

Figure 2 graphically shows the hourly averages of carbon monoxide outside our laboratory obtained with a process analyzer (Mine Safety Appliances, Pittsburgh, Pennsylvania), a commercial version of the automated gas chromatograph developed by APCO. The data was taken over a 13 week period starting 3-18-69 and terminating 6-15-69 and represents 10,709 gas chromatographic measurements. The sampling site was a point approximately 70 feet above ground level, outside the APCO laboratories in Cincinnati. The diurnal averages as measured by a NDIR analyzer located in APCO's Continuous Air Monitoring Program (CAMP) station located in downtown Cincinnati is also shown in figure 2.

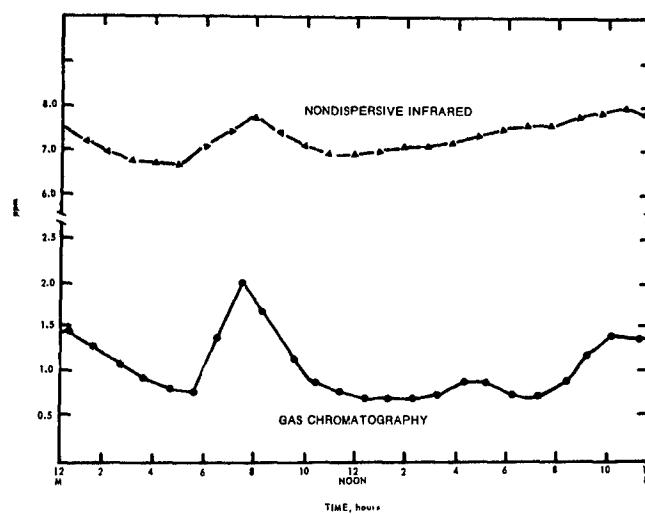


Figure 2. HOURLY AVERAGE OF CARBON MONOXIDE OVER A 3 MONTH PERIOD AT APCO LABORATORIES IN CINCINNATI, OHIO.

The hourly averages shown in figure 2 indicate that between the hours of 0530 and 1000 hours the carbon monoxide reaches a maximum concentration due to the increased morning traffic and restricted vertical mixing under a nocturnal inversion layer that often exists in the area. Between 1600 and 2000 hours a slight rise in the carbon monoxide concentration was observed, due to the homeward bound afternoon traffic. However, the average maximum value is low because of the absence of a stable inversion layer. Between 2000 and 0100 hours the carbon monoxide reaches a third maximum due to an inversion layer that begins to form as the air cools and the normal increased late evening traffic. As the traffic subsides during the early morning hours the carbon monoxide concentration, as expected, decreases.

This general diurnal pattern of carbon monoxide concentration has also been observed by our CAMP station NDIR analyzer located in downtown Cincinnati. This comparison is graphically represented in figure 2.

Figure 3 is a typical chromatogram obtained with the prototype analyzer for a prepared air sample containing less than 1 ppm methane and carbon monoxide.

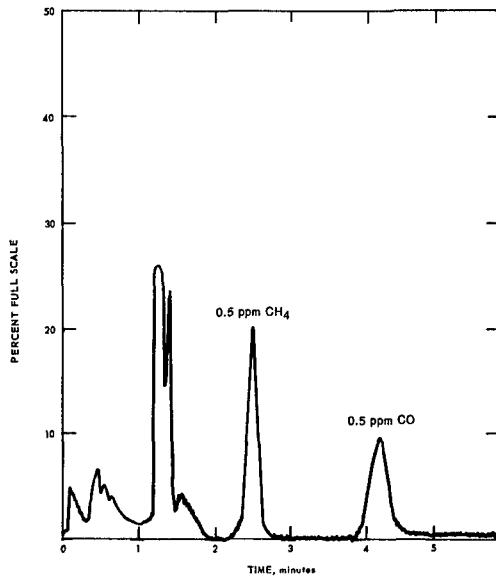


Figure 3. GAS CHROMATOGRAPHIC ANALYSIS OF CARBON MONOXIDE AND METHANE AT 0.5 PPM, V/V.

Beckman Instrument Company and Union Carbide Instrument Company have fabricated process analyzers based on the previously described developments which cannot only measure carbon monoxide and methane but also measure the total hydrocarbon content of the sample. Commercial process analyzers are designed to hold the maximum voltage signal produced by each peak within a special holding amplifier. These amplifiers can be interrogated in sequence, by a data acquisition system once every 5 minutes. The signals held on the amplifiers are updated continually as the ambient air analysis is repeated; at the same time the recorded signals are presented in a special bar mode format. This is accomplished by special circuitry which automatically zeros the baseline and preattenuates the signal for each component prior to its elution from the chromatographic column.

Process analyzers of this type are ideal for air monitoring station because their measurements can be relayed to magnetic tape through the data acquisition system, thus facilitating signal storage or relay of information. The Air Pollution Control Office has evaluated several commercial process CO and CH<sub>4</sub> analyzers and has found their performance to be superior to classical techniques. One must remember, however, that the system is a gas chromatograph and therefore requires certain skills to maintain which are often not available within local and state monitoring programs. This situation is gradually changing and state and local air pollution personnel are being trained to operate advanced state-of-the-art instrumentation.

#### B. Description of Prototype Hydrogen Sulfide and Sulfur Dioxide Monitor

Chromatograph, Detector and Sampling Systems: The system shown in figure 4 was developed to measure ambient concentrations of SO<sub>2</sub> and H<sub>2</sub>S. This analyzer consisted of a flame photometric detector, 750 volt power supply and electrometer (Micro Tek Instruments Incorporated\*, Austin, Texas), gas chromatograph (Model 1200 Varian Aerograph, Walnut Creek, California), 36 feet x .085 inch I. D. teflon column packed with 40-60 mesh teflon coated with a mixture of polyphenyl ether and orthophosphoric acid and a 6 port rotary Chromatronics automatic gas sampling valve equipped with a 10 cc teflon gas sampling loop. The flame photometric detector was equipped with a narrow band optical filter which permitted 56% light transmission at 394  $\mu$  with a band width of 5  $\mu$ . This analytical system is satisfactory for monitoring of SO<sub>2</sub> and H<sub>2</sub>S in atmospheres which do not contain significant concentrations of sulfur compounds having three or more carbon or sulfur atoms. If the atmosphere does contain such species, they could eventually elute into the detector causing positive interferences.

\*Mention of commercial products does not imply endorsement by the Air Pollution Control Office.

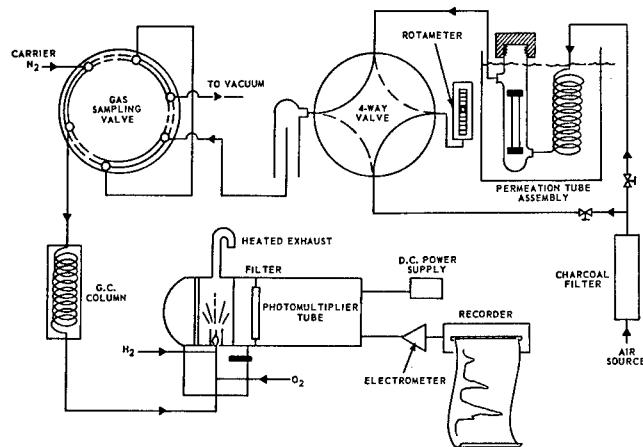


Figure 4. AUTOMATED GAS CHROMATOGRAPH-FLAME PHOTOMETRIC DETECTOR SULFUR GAS ANALYZER AND CALIBRATION APPARATUS.

To prevent the occurrence of such interferences, the chromatographic system described was modified (figure 5) by replacing the 6 port valve with a 10 port valve (Sliding plate solenoid actuated valve, Beckman Instruments, Fullerton, California) equipped with stripper column. The stripper column permitted the passage of  $\text{SO}_2$ ,  $\text{H}_2\text{S}$ ,  $\text{CH}_3\text{SH}$  and  $\text{CH}_3\text{-S-CH}_3$  to the analytical column but held back higher molecular weight compounds. This stripper column consisted of 24 inches x 0.085 inch I. D. teflon tubing packed with teflon coated with polyphenyl ether. The timing sequence used for actuating the 10 port valve for sample injection, foreflushing and back-flushing the stripper column and filling the sample loop is given below:

1. Fill sample loop.
2. Inject sample through stripper column to analytical column and maintain valve in this flow configuration for 60 seconds.
3. Actuate valve to backflush stripper for 8 minutes.
4. Return valve to foreflush position and fill sample loop for 60 seconds before injecting next sample.

Column Preparation: Columns were prepared by lightly packing 36 feet of 1/8 inch x 0.085 inch I. D. FEP tubing, at 5 C, with 40/60 mesh Haloport-F (Teflon powder, R & M Scientific, Avondale, Pennsylvania). (In practice 12 feet lengths of tubing are packed separately by filling from both ends and connecting the three columns with teflon unions.) If columns are packed too tightly, pressures in excess of 60 psi will be required to achieve the desired flow rates).

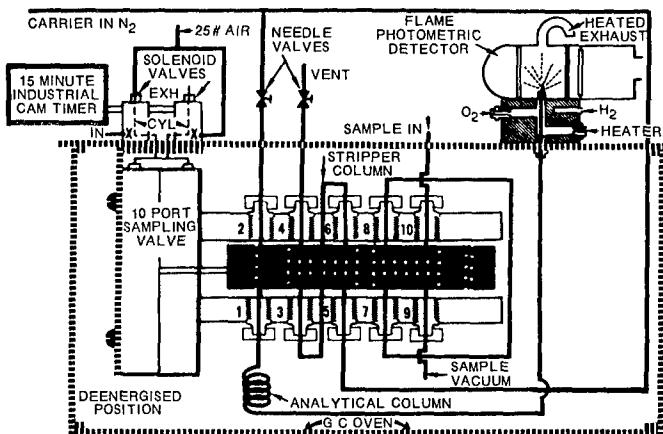


Figure 5. AUTOMATED GAS CHROMATOGRAPH-FLAME PHOTOMETRIC DETECTOR SULFUR GAS ANALYZER EQUIPPED WITH PRE-COLUMN.

Fifty milliliters of an acetone solution containing 12 grams of polyphenyl ether (5-ringed polymer, F & M Scientific, Avondale, Pennsylvania) and 500 milligrams of analytical grade ortho-phosphoric acid was forced through the 36 foot packed teflon column at a flow rate of 20 milliliters per hour under nitrogen pressure. Nitrogen was passed through the column until the column packing appeared visibly dry. This usually took 7-8 hours. The column was conditioned at 140 C for 6 hours while a nitrogen carrier gas flow of 50 milliliters per minute was maintained through the column.

Preparation of Standard Gas Samples: Teflon permeation tubes gravimetrically calibrated according to the procedure of O'Keeffe and Ortman (1966) were used as standard sources of  $SO_2$ ,  $H_2S$ ,  $CH_3SH$  and  $CH_3-S-CH_3$ . Calibration was obtained by injecting into the chromatographic column a volume of 10 milliliters containing a known concentration of the sulfur compound. Known concentrations are obtained by flowing air at a known rate over a calibrated permeation tube held at a constant temperature. The calibration system is shown in figure 1.

## RESULTS AND DISCUSSION

**Detector and Materials of Construction:** The gas chromatographic system previously described by Adams and Koppe (1967) utilized the microcoulometric titration cell. Investigations with this detector indicated that the minimum detection level for  $\text{SO}_2$  was 0.05 ppm and for  $\text{H}_2\text{S}$  was 0.1 ppm. However, severe losses of  $\text{SO}_2$  and  $\text{H}_2\text{S}$  were experienced by Koppe and Adams (1967) when the sample containing these sulfur compounds passed through a gas chromatographic column. Glass, teflon and stainless steel columns packed with a variety of solid supports and coated with a number of liquid phases were evaluated. These authors were unable to find a satisfactory combination of liquid phase, support and column construction which would permit the measurement of  $\text{SO}_2$  below 25 ppm or  $\text{H}_2\text{S}$  below 0.25 ppm. The conclusions drawn from their study indicated that it would be necessary to concentrate samples approximately 10-50 fold to determine normal ambient levels of  $\text{SO}_2$  and  $\text{H}_2\text{S}$ .

The flame photometric detector recently developed by Brody and Chaney (1966) as a gas chromatographic detector for sulfur or phosphorous compounds has shown potential as a monitoring device for volatile sulfur compounds. Studies by Stevens, O'Keefe and Ortman (1969) utilizing the FPD, suggested that in most urban environments total gaseous sulfur and sulfur dioxide were essentially synonymous. To substantiate this hypothesis, it was imperative to acquire the ability to discriminate chromatographically among the several volatile sulfur compounds which could conceivably stimulate responses from the flame photometric detector. Our approach consisted of a systematic attempt to eliminate all possible sites of adsorption or reaction between reactive sulfur compounds and the analytical apparatus.

Fluorinated ethylene propylene (FEP) tubing was found to be inert enough to pass, reproducibly, low ppb levels to the detector.

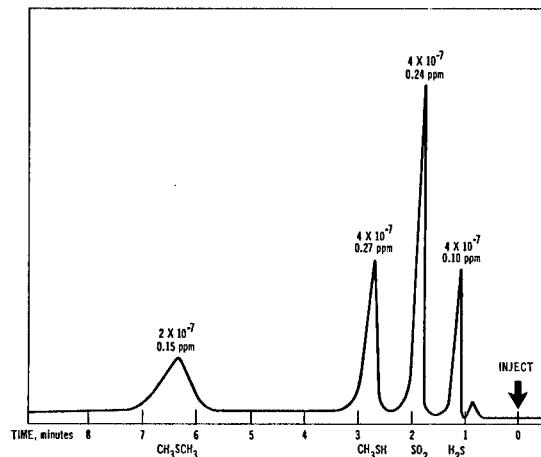


Figure 6. CHROMATOGRAMS OF A MIXTURE OF  $\text{SO}_2$ ,  $\text{H}_2\text{S}$ ,  $\text{CH}_3\text{SH}$  AND  $\text{CH}_3\text{-S-CH}_3$  AT CONCENTRATION BELOW 1 PPM, V/V.

Several liquid phases and column supports were evaluated. Polyphenyl ether on 40-60 mesh teflon was found to be a combination which would separate hydrogen sulfide and sulfur dioxide without significant loss of sample. Figure 6 is a typical chromatogram of an air sample containing sub-parts-per-billion v/v concentrations of hydrogen sulfide, sulfur dioxide, methyl mercaptan and dimethylsulfide.

### CONCLUSIONS

Automated chromatographs have been developed which have been used by the Division of Chemistry and Physics, Air Pollution Control Office (APCO), to measure ambient concentrations of a variety of pollutants including carbon monoxide, methane, sulfur dioxide and hydrogen sulfide. These systems have proved to be reliable and can operate unattended for up to 14 days.

Commercial versions of the analyzers developed by APCO are now currently available and have been evaluated on a limited scale. These studies indicate that process chromatographs can be used as a routine air pollution monitor and in some ways are superior to classic air pollutant measuring instrumentation.

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## CONSIDERATIONS IN DEVELOPING AIR QUALITY CRITERIA

F. Gordon Hueter, Ph. D.  
J. C. Romanovsky  
and  
D. S. Barth, Ph. D.

U. S. Public Health Service  
Durham, North Carolina

### Need for Air Quality Criteria

Social - The quality of the atmosphere is one important factor determining the survival of vegetation, animals and man. Through ignorance and flagrant neglect this quality has been allowed to deteriorate to the point where it now damages the biological integrity of plants and animals, and indeed the health and welfare of man himself. Wastes produced by man and his technology have begun to accumulate in the atmosphere because they are being added at a rate that far exceeds the natural processes providing for their removal. The particles, aerosols, and gases that constitute this atmospheric pollution accelerate the destruction of materials and buildings; damage crops, vegetation, and animals; and impair the health, comfort, and well-being of man. In addition, smoke, haze, smog, and undesirable odors mar the aesthetic qualities of the air. Major cities have become heat islands giving localized air pollution the potential to initiate widespread weather modifications. The deteriorating quality of the air is currently leading to an ecological crisis of world-wide proportions.

Legal - Recognition of the atmosphere as a limited natural resource pointed out the need to develop a resource-management strategy for restoring and maintaining the quality of the air at a level necessary to avoid plant and animal damage and insure the health and welfare of man. The Clean Air Act and its amendments constitute such strategy in the United States and has delegated the responsibility of implementing this strategy to the Department of Health, Education, and Welfare, National Air Pollution Control Administration. A portion of this Act requires the Secretary, Department of Health, Education, and Welfare to designate and examine intrastate air quality control regions, and to publish documents outlining criteria and associated control techniques for known air pollutants. Following the publication of each set of documents, the states with designated control regions are required to develop, in a specified period of time, respective air quality standards and implementation plans for achieving the proposed standards for those pollutants. All such proposed standards and implementation plans are subject to approval by the Secretary, Department of Health, Education, and Welfare.

## Air Quality Criteria

Purpose - Air quality criteria documents serve to initiate the establishment of state standards as well as to provide a comprehensive information source and to promote comparable judgment during the development of standards by the individual states

Definition - Air quality criteria are compilations of the latest available scientific information on the sources, prevalence, and manifestations of recognized air pollutants. Most important, they describe what effects have been associated with, or may be expected from, an air pollutant level in excess of a specific concentration for a specific time period. Such effects generally involve visibility reduction, damage to materials, economic costs, vegetation damage, nuisance aspects, and adverse effects on the health and well-being of man and animals.

In comparison, an air quality standard establishes pollutant levels that cannot legally be exceeded during a specific time span, in a specific geographic area. In short, air quality criteria are descriptive, whereas air quality standards are prescriptive.

Factors considered - Air quality criteria documents are planned to provide comprehensive information in the following major areas whenever pertinent:

### I. Environmental Appraisal

- A. Origin (natural and man-made) and fate
- B. Physical and chemical properties
- C. Spatial and temporal distribution in the atmosphere
- D. Atmospheric alterations
  - 1. Chemical transformations
  - 2. Meteorological influences
- E. Measurement technology

### II. Effects on Human Health (including laboratory animal studies)

- A. Toxicologic appraisal
  - 1. Behavioral and sensory responses
  - 2. Biochemical and physiological mechanisms and responses
- B. Epidemiological and clinical appraisal
  - 1. Field studies
  - 2. Clinical studies

### III. Other (welfare) Effects

- A. Biological and physical effects (including significant microfauna and flora)
  - 1. Plant life (natural and cultivated)
  - 2. Domestic and wild animal life
  - 3. Materials
- B. Social and aesthetic effects

- C. Economic impact (includes that resulting from all other effects listed on the previous page plus effects on specific economic parameters such as real property values, etc.)

#### IV. Gap Areas in Current Knowledge

##### Limitations of Air Quality Criteria

There are several general areas of knowledge where information is usually inadequate, and because of the nature of their shortcomings, they merit attention only to point out that our knowledge of them may never be complete.

1. Effects related to exposures to ambient air can never take into account the presence of all of the pollutants which may be causing the net result; some of the pollutants may be intermediate or unstable products of known substances, and the presence of others may be unsuspected, and thus not even monitored.
2. Laboratory experiments using simulated air pollution cannot totally replicate the actual ambient air in composition, temperature, and humidity, simultaneously, both because of the constant fluctuations of these variables in the ambient air and because of the presence of other pollutants (some not routinely measured), which may be contributing to the effects observed.
3. Dose-response relationships between the ambient air and observed effects can only be estimated, since the actual dosage is only an estimate or average of an exposure, which is often extremely variable over relatively short periods of time.
4. It is extremely difficult, if at all possible, to state minimum or threshold levels for a particular pollutant with reference to a particular effect. Long-term exposures allow too many variables to exert an additional influence on the outcome of a particular observation; short-term exposures tend to rely upon subjective evaluations, thus tend to depend, also, on the subjects' preparation and experience, personal opinion, on the bias of both the investigator and the subject, and on other environmental variables which cannot be well controlled.
5. Most studies of effects are not directly comparable with each other because of non-parallel exposure times or conditions and because of variation in measurement technique and averaging times.
6. Air quality data often do not reflect the actual exposure of the subjects being studied.

The protection of public health requires action based upon best evidence of causation available. This philosophy was appropriately expressed by Sir A. B. Hill (1962) when he wrote:

"All scientific work is incomplete-whether it be observational or experimental. All scientific work is liable to be upset or modified by advancing knowledge. That does not confer upon us a freedom to ignore the knowledge we already have, or to postpone the action that it appears to demand at a given time."

Even though we cannot quantify with great precision the health and welfare effects of air pollution we maintain that until better measurements are possible our actions must be based upon the best knowledge we have and be guided by the principle of the enhancement of the quality of human life. Such action is based on a philosophy of preventive medicine.

#### Formulation and Review

As required by the Clean Air Act, a National Air Quality Criteria Advisory Committee was established with a membership representing industry, universities, local and state governments. The Committee provides advice concerning policy and procedures under which to issue criteria, and contributes assistance in drafting the documents.

Expert consultants and staff members of NAPCA provide first drafts of portions of each document. The drafts are then subjected to a sequence of extensive reviews and revisions by the committee and other individual reviewers, who are deemed particularly competent in the fields of science and technology related to the problems of the specific atmospheric pollutants under investigation. In addition, the Clean Air Act, as amended, requires review of each document by appropriate Federal departments and agencies and subsequent mutual discussion of their comments. The final responsibility for the contents of each document lies with NAPCA.

#### Revisions

With the continuing growth in the knowledge of the effects of air pollutants on biological systems and with the advances in the technology of air pollution surveillance, air quality criteria need periodic review to determine whether earlier conclusions should be modified. At least once every five years, each criteria document will be considered regarding the nature and magnitude of the necessary revision.

#### Summary

In order that adequate background material be available for consideration by state officials responsible for adopting ambient air quality standards, the criteria documents

are formulated to provide such comprehensive information. Air quality criteria, therefore, are expected to describe the concentrations, exposure times, meteorological interactions, and other potentiating influences that are associated with health, welfare, or economic effects attributable, either directly to the contaminant in question, or to some analog of that contaminant. Air quality criteria include measurement methodology for rigorous application in determining the air quality. They also provide information on chemical contaminants in order to facilitate the rational development of control strategies. Air quality criteria issued to the states are required by law to be reviewed by the scientific community and by other Federal agencies. The scientific community is primarily reflected in the National Air Quality Advisory Committee, made up of as many as fifteen authorities in universities, industry, local and state governments. The criteria documents are additionally reviewed by a large number of scientists outside of the committee.

## DISCUSSION

DR. MAC EWEN: Dr. Hueter, I'd like to ask you two related questions: 1) What criteria have been published, and you also indicated that in addition to publishing criteria that effective methods of reduction of pollutant would also be published, and 2) have any of those been published?

DR. HUETER (Environmental Health Service): The criteria documents and companion control technology documents have been published for  $\text{SO}_X$ , particulates, carbon monoxide, oxidants, reactive hydrocarbons, and presently it is planned by the end of this calendar year that there will be documents for lead, fluoride, polynuclear organic matter, and  $\text{NO}_X$ , nitrogen oxides. This is a tentative schedule and we're doing everything in our power to meet that schedule. We're working very closely with the National Academy of Sciences in providing initial portions of many of these documents and it is planned that they will be published early next calendar year.

DR. MAC FARLAND: You mentioned that these documents have to be kept under continual review and that there is an intention to perhaps issue them in a new addition at least every five years. With regard to the individual pollutants that have been reviewed, and thinking of the varying research activity with respect to specific ones of these, is there any possibility that a given document, let's say for purposes of argument, the  $\text{SO}_X$  document, might conceivably come up for review, let's say two years from now, three years from now?

DR. HUETER: Yes, that's one reason I said at least every five years. If there is some scientific breakthrough which would indicate that there is a really serious effect that had not been recognized earlier, these documents would be immediately modified to take into account that information.

DR. THOMAS: One comment. At many previous conferences I mentioned, I think, that the work we were doing primarily for the reason of continuous exposure to space cabin atmosphere, that much of these data, have quite a bearing on setting clean air limits. We were just reviewing the draft of your  $\text{NO}_2$  document and one of the first recommendations for research to be performed is continuous exposures to  $\text{NO}_2$ . We'll be glad to hand you 90-day continuous exposure data and two-week continuous exposure data and some tentative limits we suggested for space cabin use. Now, we didn't feel that the use of an exotic atmosphere with 5 psi oxygen had any real interference with the  $\text{NO}_2$  effect, either positive or negative but believe me that was a lot of work at various dose levels and we'll be glad to supply the documents if you want them.

DR. HUETER: We'd be extremely grateful to receive them, I assure you. And they will be utilized.

DR. BEARD: You mentioned the requirement of establishing standards and a control program within a stated period after publication of a criterion document. How exact or how mandatory is this? I'm thinking in particular of the publication of the hydrocarbon document which in the view of some of us in California to set a standard for hydrocarbon is a redundancy; one sets a standard for oxidant. Are we going to be compelled to have a standard for hydrocarbon even if we think it's a redundancy?

DR. HUETER: I certainly would not speak for Congress or for the Administration for what you will and will not be compelled to do. Obviously this is not my area of knowledge, but I do know the law does state that there are 90 days in which to file intent to set standards, 180 days to hold hearings and set standards, and another 180 days to file your implementation plans which explains how you are going to achieve those standards even, recognizing in many cases it will be a step wise achievement but I really don't know what new legislation has in store for us, it may change the whole picture.

DR. BACK (Aerospace Medical Research Laboratory): Since I just looked over the NO<sub>X</sub> document for the Air Force and didn't have very much time to do it, unfortunately, two questions came to mind. One of them is, these are voluminous documents and there are many parameters looked at. There are soil microorganisms looked at, etc., etc. My first question is, have any standards been set from the criteria from one document or from any document, for the pollutants?

DR. HUETER: There have been standards set and approved in relation to SO<sub>X</sub> in particular. I can't quote what they were exactly, in what states they were approved or where they weren't.

DR. BACK: Were these based on the most susceptible organisms, on the human data on the animal data or what? In other words what data was primary in setting the standard?

DR. HUETER: In my opinion it was the health effects data.

DR. BACK: On humans?

DR. HUETER: Yes.

DR. LEON: I'd just like to know as labs or as individuals how we can get, make sure that we get, these documents? How do we get on the mailing list?

DR. HUETER: These documents are sent out by Air Pollution and Technical Information Center, abbreviated as APTIC which is located in the, well presently Durham, North Carolina would be fine as an address, NAPCA in Durham. They'll be moving soon to a new facility, so that's why I hesitate to give an address.

DR. DU BOIS: A couple of comments; Dr. Leon's request, I think, should be reciprocated in the sense that I'm sure NAPCA would like to have reports on the work done at your lab and also here at this Base. The retrieval system is such that the search through the Smithsonian goes back for I think only one year or perhaps two years on their governmental filing of research in progress, so that for example, work done here five years ago or ten years ago might miss this dragnet and might not get into the NAPCA document unless they were indexed in the world literature and many reports would not be, or unless somebody just happened to have one in his drawer and knew about it. So this is one place you can help. The other point was that Ralph Wands sent me a reprint from Science on someone who had pointed out areas of common interest between the NAPCA program or more generally the interest in human health and welfare compared to the space program and its interest in ecological systems for manned flight of long duration. There are many areas of common interest such as instrumentation, health effects, and I hope that in the planning of future programs, these organizations can get together on working out their programs to take advantage of these areas of "commonality".

DR. JACOBSON (Tulane University School of Medicine): I should explain to those of you who don't know, that Dr. DuBois is Chairman of the National Academy of Sciences Committee which is primarily responsible for the preparation of the criteria documents for NAPCA.

## GAS CHROMATOGRAPHIC RETENTION DATA

William H. Toliver, Sr.

Sheldon A. London, Ph. D.

and

Anthony A. Thomas, M. D.

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

### INTRODUCTION

Gas chromatography (GC) is one of the more useful instrumental methods of analysis in biochemical research (Burchfield and Stores, 1962) for toxicology (Kazyak and Knoblauch, 1963) and pollution studies (Altshuller, 1963). Yet, reproducibility is one of the more pressing problems that prevent the ready use of this analytical method. Retention data are difficult, and often times impossible, to reproduce from laboratory to laboratory, from gas chromatograph to gas chromatograph, or indeed on the same gas chromatograph.

This paper will indicate an approach to the solution of the problem of reproducibility of retention data. The main purpose is to present a method of reporting gas chromatographic data that is reproducible, meaningful and relatively easy to generate. In addition, two questions will be discussed: 1) What is retention data? and 2) What value does it have for the toxicologist, indeed to all users of gas chromatography?

### Chromatogram

A chromatogram usually indicates a starting point, an air peak, and a solute peak(s) as shown in figure 1. The following definitions stress the difference between the total elution time value and the characteristic time value:

AB = Retention time for mobile phase ( $t_M$ )

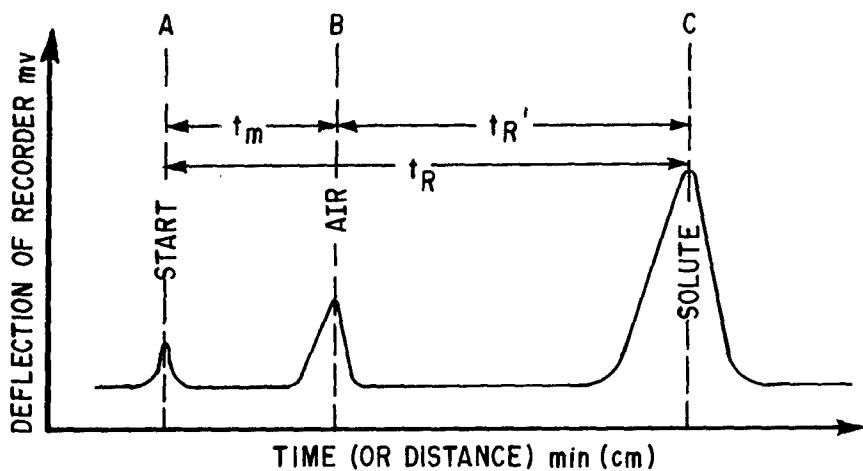
AC = Uncorrected retention time ( $t_R$ )

BC = Adjusted retention time ( $t_R'$ )

$$= AC - AB = t_R - t_M$$

Thus, the term "retention time" is not definitive since a distinction must be made between the total time ( $t_R$ ) and the characteristic value ( $t_R'$ ), the adjusted retention time. The product of the flow rate ( $F$ ) and the respective retention time gives the corresponding retention volume.

Most toxicologists use the term "retention time" which does not indicate if  $t_R$  or  $t_R'$ , or both are intended. In order to be meaningful, adjusted retention time ( $t_R'$ ) or volume ( $V_R'$ ) must be corrected and related to specific experimental conditions; i. e., the compressibility of the carrier gas, the influence of the gas laws, pressure, temperature and flow rate. However, after making the above operating corrections, a more difficult problem arises because these operating variables change without notice, sometimes even during a run. So we must find a more satisfactory method than  $t_R'$  or  $V_R'$  to express retention data. We shall consider the kinds of retention data in order to make the most satisfactory selection.



$$AB = t_M \text{ (MIN)}$$

$$t_M \times F = V_M \text{ (ml)}$$

$$AC = t_R$$

$$t_R \times F = V_R$$

$$BC = t_R'$$

$$t_R \times F = V_R'$$

Figure 1. TYPICAL CHROMATOGRAM OF SINGLE SOLUTE

Classification of Retention Data

Qualitative gas chromatographic values (table I) may be expressed as absolute retention values or as relative retention values. The former group are expressions of some function of retention time and the latter are expressions of the retention relative to some standard(s).

TABLE I  
CLASSIFICATION OF RETENTION DATA

- I. Absolute Retention Values
  - a. Retention time ( $t_R$ )
  - b. Retention volume ( $V_R$ )
  - c. Specific retention volume ( $V_g$ )
  - d. Partition coefficient (K)
- II. Relative Retention Values ( $\alpha$ )
  - a. Nonane values ( $R_{X9}$ )
  - b. Retention Index (I)

In the first group are retention time ( $t_R$ ) retention volume ( $V_R$ ), specific retention volume ( $V_g$ ) (Littlewood, A. B. et al, 1955), and partition coefficient (K).

$V_g$  and K are exact, fundamental values; however, they are not commonly used. Their calculation requires knowledge of variables that are not generally known, that are difficult to measure, and that may change in use; they have little descriptive value. Since  $V_g$  is calculated at 0 C, it does not indicate the temperature dependence of retention and it is not possible to determine exact values for them with normal commercial instruments.

Thus the second group, relative retention data, enjoys wide use among gas chromatographers. However, it is difficult to express the temperature dependence of the values and it is practically impossible to fix only one standard as in the theoretical nonane values (Evans, 1961).

To overcome these difficulties, Kovats in 1958 proposed an improvement of the application of qualitative gas chromatography. A substance is characterized by the logarithm of its retention, rather than by a relative time value. The values obtained are related to a range of the retention values of n-alkanes and not to only one reference substance.

TABLE II  
COMPARISON OF RETENTION VALUES

<u>Variables</u>	<u>Absolute*</u>	<u>Relative</u>
Type of Stationary Phase	yes	yes
Temperature	yes	yes
Column length (amount)	yes	no
Pressure drop	yes	no
Flow rate	yes	no

\*Experimental conditions are almost impossible to reproduce from day to day or run to run, or even on same instrument.

Comparison of retention values (table II) indicates that the relative values are functions of fewer variables (only type of stationary phase and temperature) than the absolute values and are therefore preferable. Because the retention index values are not dependent on only one standard it is preferable to the nonane system. The remainder of this paper will examine the Kovats Retention Index.

#### Definition of Kovats Retention Index

In the Kovats (1958) index system (I) the position of the eluted substance is indicated on a scale obtained from the gas chromatogram. The points of reference on this scale are the values of the general formula, i.e.  $-C_Z H_{2Z+2}$  (Z is carbon number) and are always defined as 100z. This can be expressed as:

$$(1) \frac{St}{T} \frac{Ph}{(n - C_Z H_{2Z+2})} = 100z \text{ and described as follows:}$$

Homologous Series  $C_Z H_{2Z+2}$  :  $H_2$ ,  $CH_4$ ,  $C_2H_6$ ,  $C_3H_8$  . . .

Retention Index 100z 0 100 200 300 . . .

Thus "I" for the n-paraffins will be 100 times the carbon number. The logarithm of  $V_g$  of the n-paraffins increases linearly with chain length and therefore the retention index scale is linear (Ray, 1954). The retention index of any substance X in isothermal GC is determined by logarithmic interpolation between the two relevant n-paraffins (Eq. 2).

$$(2) I_T^{\text{St Ph}} = 100 \frac{\log V_g(x) - \log V_g(P_z)}{\log V_g(P_z + 1) - \log V_g(P_z)} + 100_z$$

$$\text{where } V_g(P_z) \leq V_g(X) \leq V_g(P_z + 1)$$

Thus, the retention value for the substance being calculated lies between two adjacent n-paraffins. In the calculation of "I", specific retention volume ( $V_g$ ) can be replaced by adjusted retention time  $t_R'$ . However, the sample must be analyzed under identical conditions as the n-paraffin references or, preferably, the references should be included in the sample being run.

Figure 2 is a graphic calculation of retention index and helps us understand its merits. Line (A) represents the chromatogram with the adjusted retention times ( $t_R'$ ) of the n-paraffins used as fixed reference points and two sample components, cyclohexane and toluene. On line (B)  $t_R'$  is plotted on a log scale. The distance between two consecutive n-paraffins is divided into 100 equal units, giving rise to line (C), and the "I" values, 690 i. u. for cyclohexane and 730 i. u. for toluene where i. u. stands for index units. The "I" values are thus descriptive, indicating where on the index scale the compound is found.

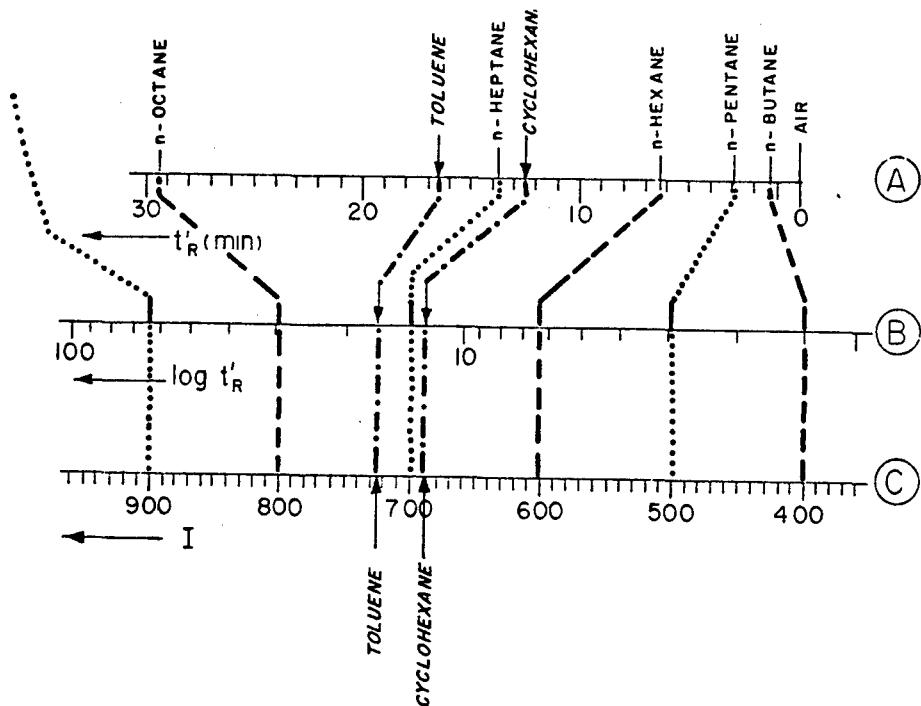


Figure 2. GRAPHIC CALCULATION OF RETENTION INDEX\*  
 \*(Ettre, L. S., presented at the Fourth International Symposium on Advances in Gas Chromatograph, Houston, Texas, June 1-4, 1964).

With the assignment of a value of zero for "I", i.e., when hydrogen is taken as "zero paraffin" ( $C_0 H_{20+2}$ ), the index region from 0-100 is available. Thus practically all substances can have an "I" value although the linearity in this region is poorer.

Since "I" is dependent only on temperature and the chemical nature of the liquid phase we shall examine the correlation between "I" and temperature, and "I" and molecular structure.

### Temperature Dependence

The relationship between the logarithms of  $V_N$  or  $t_R'$  and the reciprocal of the absolute column temperature is linear (Purnell, 1962). However, "I" is directly proportional to column temperature and in a first approximation this correlation is linear (table III). The "I" values that correspond to the intervals between temperatures ( $\partial I / \partial T$ ) are relatively small and linear for both the apolar column,  $I^A$ , Apiezon L, and the polar column,  $I^P$ , Emulphor - O; thus "I" values at different temperatures are predictable and can be calculated.

TABLE III  
RETENTION INDEX FOR LIMONENE AT VARIOUS TEMPERATURES

#### Various Temperatures

Temperature °C	$I^A$	$\partial I / \partial T$	$I^P$	$\partial I / \partial T$
150	1067		1174	
170	1074	7	1178	4
190	1081	7	1183	5
210	1178	8	1188	5

### Nomenclature

By convention, "I" indicates retention index (table IV). The temperature and stationary phase are indicated; thus 1,8 cineol determined on Apiezon L at 190°C is noted as  $I^A_{190}$  Apiezon L = 1079. When reporting a compound for the first time, a range of temperatures in which "I" has been determined is given so that the temperature coefficient for ten degrees ( $10 \partial I^A / \partial T$ ) can be reported; it is 5.0 for the compound above "I" and

$(10^3 I / {}^\circ T)$  are usually reported on at least two different types of stationary phases, apolar (A) and polar (P). Data in this form permit interpolation in the given range and some modified extrapolation beyond the range.

TABLE IV

TEMPERATURE COEFFICIENTS OF SOME RETENTION INDICES ON APEZON L ( $I^A$ ) AND EMULPHORE - 0 ( $I^P$ )

<u>Solute</u>	Temp Range (°C)	$I^A_{190}$	$10 \frac{\partial I^A}{\partial T}$	$I^P_{190}$	$10 \frac{\partial I^P}{\partial T}$
1-8 Cineol	170-210	1079	+ 5.0	1199	+ 6.3
Methyl Ether of Thymol	190-230	1233	+ 2.7	1472	+ 3.5

The difference between two "I" values can be expressed on one stationary phase ( $\Delta I$ ) or two stationary phases ( $\Delta I$ ). The former can indicate either the difference of "I" for one substance at two temperatures or the difference of "I" for two substances at one temperature. The latter,  $\Delta I$ , is given at the same temperature.

Relationship Between "I" and Molecular Structure

Kovats et al have formulated a set of rules based upon the regularity of the empirical data. The first four rules deal with "I" values determined on one stationary phase.

Rule 1 - IN ANY HOMOLOGOUS SERIES THE RETENTION INDEX OF THE HIGHER MEMBERS INCREASES BY 100 i. u. PER  $CH_2$  GROUP INTRODUCED (Kovat and Wehrli, 1958, 1959, 1961).

As shown in table V, methyl ketones above pentanone-s for polar and apolar stationary phase give increments of 100.

Rule 2 - IF THE DIFFERENCE IN THE BOILING POINT OF TWO ISOMERS IS  $\Delta t_b$  THEN THE DIFFERENCE IN THEIR RETENTION INDICES, ON A "NON POLAR" STATIONARY PHASE IS GIVEN BY

$$(3) \quad \Delta I \approx 5 \times \Delta t_b$$

(Kovats and Wehrli 1958, 1959, 1961)

TABLE V  
RELATIONSHIP BETWEEN I AND MOLECULAR STRUCTURE

<u>Methyl Ketones</u>	<u><math>I_{130}^A</math></u>	<u><math>I_{130}^P</math></u>
1. Acetone	450	708
2. Butanone-2	551	792
3. Pentanone-2	644	878
4. Hexanone-2	747	979
5. Heptanone-2	846	1079
6. Octanone-2	947	1179
7. Nonanone-2	1047	1277
8. Decanone-2	1148	-
9. Undecanone-2	1247	-

"Non Polar" stationary phases as distinct from apolar, are defined as pure paraffins or mixtures of pure paraffins.

Solution of Equation 3, the gas chromatographic equivalent of Trouton's Rule, from the values presented in table VI gives  $\alpha I \approx 5 (144.4 - 139.1) + 877 \approx 904$  i. u.

TABLE VI  
GAS CHROMATOGRAPHIC EQUIVALENT OF TROUTON'S RULE

<u>Solute</u>	<u><math>I_{78}^A</math> C</u>	<u>Boiling Point (°C)</u>
o-xylene	X	144.4
m-xylene	877	139.1

$$X = 904 \text{ i. u. (899)}$$

The observed value is 899 i. u.; this represents less than a 1% error from the experimental value which is close enough for molecular structure elucidation.

Rule 3 - THE RETENTION INDICES OF ANY ASYMMETRICALLY SUBSTITUTED COMPOUND CAN BE CALCULATED FROM RETENTION INDICES OF THE CORRESPONDING SYMMETRICALLY SUBSTITUTED SUBSTANCES (Evans and Smith, 1961).

Example:

$$I_{130}^{Ap-L}(R, R') = \frac{547 + 855}{2} = 701 \text{ i. u.}$$

The values for the two symmetrically substituted compounds (R, R) and (R', R') were 547 and 855 respectively. The calculated value 701 i. u. is less than 1% off from the experimental value 693. This is close enough for molecular structure elucidation.

Rule 4 - SIMILAR SUBSTITUTION IN SIMILARLY CONSTITUTED COMPOUNDS INCREASES THE RETENTION INDICES BY THE SAME AMOUNT FOR A GIVEN STATIONARY PHASE (SWOBODA, 1962).

Table VII gives a list of primary (1, 2, 3), tertiary (4, 5), and aromatic primary (6) alcohols. The acetylation of these alcohols gives acetates whose average increment ( $\Delta$  i. u.) between acetate and parent alcohol is 103 i. u.

TABLE VII  
RETENTION INDICES FOR SOME ALCOHOLS AND THEIR ACETATES

Compound	Alcohol $I_{190}^A$	Acetate $I_{190}^A$	$\Delta$ i. u.
1. citronellol	1188	1290	102
2. nerol	1192	1292	100
3. geroniol	1210	1312	102
4. decanol-1	1244	1353	109
5. linalool	1076	1184	108
6. benzyl alcohol	1046	1145	99

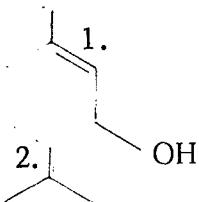
The previous four rules summarize the empirical regularities in retention indices of a group of substances on one stationary phase. The two following rules deal with the comparison of the retention indices of the same substance on different stationary phases.

Rule 5 - (a) THE RETENTION INDICES OF "NON-POLAR" SUBSTANCES REMAIN ALMOST CONSTANT FOR ANY KIND OF STATIONARY PHASE. (b) THE RETENTION INDICES OF ANY SUBSTANCE ARE ABOUT THE SAME FOR ALL "NON-POLAR" STATIONARY PHASES (Kovats and Wehrli, 1958, 1959, 1961).

Rule 5a and 5b deal with "non-polar" substances on different stationary phases and different substances on "non-polar" stationary phases. Examples of 5a are the "I" values for 2-methylpentane on hexatriacontane (568 i. u.), hexadecane (569 i. u.), squalane (570 i. u.), silicone oil (568 i. u.), dinonylphthalate (570 i. u.) and benzylidiphenyl (569 i. u.). The first and last "I" values were obtained at 78 C; all others were determined at 50 C. The polarity of the above stationary phases increases progressively as listed.

Rule 6 - IF A SUBSTANCE CONTAINS MORE THAN ONE ADHERING ZONE, THEN THE  $\Delta I$  VALUE OF THIS SUBSTANCE CAN BE CALCULATED BY ADDING UP THE INDIVIDUAL INCREMENTS FOR EACH OF THE ADHERING ZONES (Kovats et al).

Example: Nerol



Hydroxyl group	367 (-2.5/10 C)
1st double bond	24 (+0.3/10 C)
2nd double bond	31 (+0.4/10 C)
<hr/>	
Calculated	422 (-1.8/10 C)
Experimental	419 i. u. (-1.9)/10 C

The relevant adhering zones have been previously calculated and tabulated. The adhering zones result from  $\Delta I$  calculations and are almost equivalent in concept to functional groups. In the example nerol, a monoterpene alcohol, at 130 C the sum of the three adhering zones, calculated without running the compound, 422 i. u., represents less than a 1% error when compared to the experimental value of 419 i. u. The numbers in brackets are variations of increment for a temperature increase of 10 C. Thus it is possible to calculate "I" for a compound run on columns for which adhering zone data have been tabulated previously.

## DISCUSSION

Accuracy and Errors

Since the retention index is a tabular system, the accuracy and qualitative specificity will be considered. In the definition of the index system we arbitrarily divided the distance between two successive n-paraffins into 100 units. More than 100 organic compounds may fall into that range and more than one compound can have the same i. u. on a given column. An example by Ettre will be instructive:

Let us suppose that a substance has a retention index of 1026. If the accuracy is 3 i. u., then we can exclude all compounds with indices above and below the 1024.5 and 1027.5 range. If 1000 compounds have retention indices between 1000 and 1100, we could exclude about 960, i. e. 96%, and have a choice of only 40 compounds. Since it is customary to measure the index on an additional column of different polarity, presumably the possibilities can be narrowed to a few choices.

This example is instructive concerning the importance of the accuracy of the measurement of "I". If the accuracy were only one instead of three units then the first determination would reduce the possibilities to only ten substances; 0.5 unit accuracy reduces the possibilities to 5 compounds, etc. Therefore we try to reduce the errors in the measurement of the index in the following ways:

1) Small samples are used to reduce errors resulting from the GC partition process, i. e., deviations from the linear distribution isotherm and the fluctuations in temperature and gas flow during partition. 2) Measurements and controls of column temperature and carrier gas flow rate are performed accurately. 3) Pure and unchanging stationary phases are used. Slight changes in chemical composition of the phase can significantly alter its retention characteristics. Special provision must be made to remove traces of oxygen from carrier gases because of the danger of solvent oxidation and subsequent change in polarity of the liquid phase. 4) Incompletely resolved peaks can be handled by the ingenious method of examining peak maximum to determine purity (Littlewood et al, 1955) or the method of computer resolution by Littlewood et al, 1970. These are some sophisticated approaches to the problem of impure peaks; however, a multicolumn determination should solve 95% of the peak resolution problems if the molecular structures of the compounds to be resolved are significantly different. For compounds whose structures are very similar, i. e., isomeric, new liquid crystal phases promise help (Kelker, 1959). 5) Solid supports that are not inert can cause retention error, particularly with polar samples. This laboratory uses Chromosorb G - H. P.

## SUMMARY

The Kovats Retention Index System relies on the basic approach of analytical chemistry of bracketing an unknown with two standards; however, in this instance the bracketing reference materials have been extended to include a homologous series whose retention provides a linear scale. Similar special systems have been derived by Woodford, Van Gant, Miva and Vanden Heuvel and Horning. The Kovats system has the advantage that it can describe on its scale any compound that can be chromatographed.

The Kovats Retention Index System has been approved and recommended by the J. Gas Chromatography and the Data Sub-Committee for the Publication of Retention Data, 1965. This general index system recommends itself for use in the expression of retention data in toxicological studies because:

- 1) It is relatively easy to obtain instrumentally. Gas chromatographs are relatively cheap and technicians are relatively easy to train.
- 2) It is relatively accurate. It is competitive with the other instrumental methods in the Silverstein approach to identification of compounds.
- 3) It is reproducible and its temperature variation is small and predictable.
- 4) The index number generated is meaningful. It can also be used in theoretical calculations.

For the above reasons we recommend the use of Kovats Retention Index System for reporting retention data in toxicological studies.

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EFFECTS OF INHALATION OF FREON 113 ON LABORATORY ANIMALS

Vernon L. Carter, Major, USAF, VC

National Aeronautics and Space Administration  
Houston, Texas

Paul M. Chikos, M.D.

14355 38th Street, N.E.  
Seattle, Washington

James D. MacEwen, Ph.D.

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio  
and  
Kenneth C. Back, Ph.D.

Aerospace Medical Research Laboratory  
Wright-Patterson Air Force Base, Ohio

Freon 113 (1, 1, 2-trichloro 1, 2, 2-trifluoroethane) is one of the recent ethanes to find wide industrial application as a refrigerant, solvent, and cleaning agent. Toxicity studies have shown it to be relatively nontoxic, and the American Conference of Governmental Industrial Hygienists<sup>1</sup> has assigned a Threshold Limit Value (TLV) of 1000 ppm. The primary response to exposure appears to be alteration in central nervous system function with concentrations of 2500 ppm and above inducing deterioration in psychomotor performance in humans (Stopps, 1967).

This presentation will cover the results of two recently completed studies on the toxicology and pharmacology of Freon 113. The first study was designed to determine the biological effect level for inhaled Freon 113 under continuous exposure conditions, for subsequent long-term experiments. The exposure concentration chosen was 2000 ppm for a period of 14 days. The second study was designed to determine the effects

<sup>1</sup>Threshold Limit Values of Airborn Contaminants Adopted by ACGIH for 1970.

of Freon 113 on transmission through autonomic ganglia. The experimental method selected for this study utilized the stellate ganglion of the spinal dog with the increase in heart rate during pre- or postganglionic stimulation serving as the end organ response. This preparation was utilized by Garfield et al (1968) for determining the degree of nicotinic and muscarinic blockade induced by certain anesthetics. It was selected by us in an attempt to compare the efficacy of Freon 113 as a ganglionic blocking agent with that observed by halothane.

## METHODS

### 14 Day Exposure

The experimental and control groups of animals were comprised of 4 monkeys and 8 dogs in each group; 40 mice and 50 rats were used in experimental groups, and control groups consisted of 20 mice and 25 rats. Monkeys and dogs were females while rats and mice were males. The exposure was conducted in a Thomas Dome at ambient conditions.

The "Freon 113 TF" used in this study was purchased from the E.I. DuPont De Nemours Company, Inc., Wilmington, Delaware. Freon 113 contaminant analysis was made by gas chromatography as described by MacEwen and Vernot (1970).

### Ganglionic Study

Female beagle dogs weighing from 8.0-13.7 kg were used in this study. The methods for preparing the animals were the same as those described by Garfield et al (1968). Briefly, they consisted of anesthetizing the dog with thiopental sodium (approximately 30 mg/kg intravenously), ligating both carotid arteries and severing both vagosympathetic trunks in the midcervical region, severing the spinal cord at the atlanto-occipital junction, and destroying the brain stem with a probe. This procedure eliminated the necessity for any further drug anesthesia. The chest was then opened with a midline incision, the right stellate ganglion isolated, and stainless steel electrodes placed on the pre- and postganglionic nerves to and from the ganglion. The heart rate was recorded from pericardial electrodes. Arterial pressure and arterial samples for blood gas analysis were obtained through a catheter placed in the femoral artery. A Statham model P23 pressure transducer was used to measure aortic blood pressure which was recorded with the heart rate on a Grass model 5D polygraph. Mean arterial pressure was maintained above 50 mm Hg by intravenous infusion of 6% dextran as needed.

Ventilation with 100% oxygen was maintained with a Harvard respiration pump. Rate and tidal volume were adjusted to maintain arterial blood oxygen partial pressure at 500 mm Hg or above. The 2% Freon 113 was prepared by vaporizing a predetermined amount of the Freon into a set oxygen flow and delivering the mixture into a reservoir. The contents of the reservoir were then delivered to the dog by the Harvard pump. The reservoir mixture was periodically checked by gas chromatography to assure that a 2% mixture was actually being delivered to the dog.

Square wave stimuli of 1 msec duration were delivered to the nerves by a Grass model S4 stimulator through a stimulus isolation unit. The experimental procedures followed were the same as those described by Garfield et al (1968) with one major exception. After supramaximal voltages had been determined, control increases in heart rate were recorded following stimulation at 1 and 3 cps for each of the pre- and postganglionic nerves. Inhalation of 2% Freon 113 was then initiated. Ten minutes was then allowed to elapse and the heart rate response to both pre- and postganglionic stimulation at all frequency ranges was determined (0.3 to 40.0 cps preganglionic and 0.1 to 10.0 cps postganglionic). The Freon 113 exposure was then terminated, 15 minutes allowed to elapse, and control heart rate responses to both pre- and postganglionic stimulation determined. This procedure of obtaining the control observations after the exposure prevented the possibility of confusing the action of the test compound with that reduction in response observed in a deteriorating preparation.

A three factorial analysis of variance was used to determine the statistical significance at the 5% level between the control and exposure observations as well as between the exposure and exposure plus atropine observations.

## RESULTS AND DISCUSSION

### 14 Day Exposure

The changes observed in animals exposed to 2000 ppm of Freon 113 for 14 days were all minimal, and could not be related to the toxic effects of the compound. Enlarged thyroid glands were observed in all rhesus monkeys exposed. Rat kidneys were the only organs showing an increase in weight over control values. These differences were minimal, and could not be conclusively attributed to the exposure.

### Ganglionic Study

The ganglionic effects produced by inhalation of 2% Freon 113 in 4 dogs are seen in figures 1 and 2. Two percent Freon 113 significantly reduced the increase in heart rate produced by preganglionic stimulation at all frequencies from 0.3 to 4.0 cps. The exposure had no effect on the heart rate response to postganglionic stimulation. Further reduction in the response to preganglionic stimulation was seen at stimulation frequencies of 1, 3, 6, 10 and 40 cps by the administration of atropine, 0.05 mg/kg intravenously. This increase in the depression by muscarinic blockage during Freon 113 inhalation could be indicative of nicotinic blockage in the stellate ganglion.

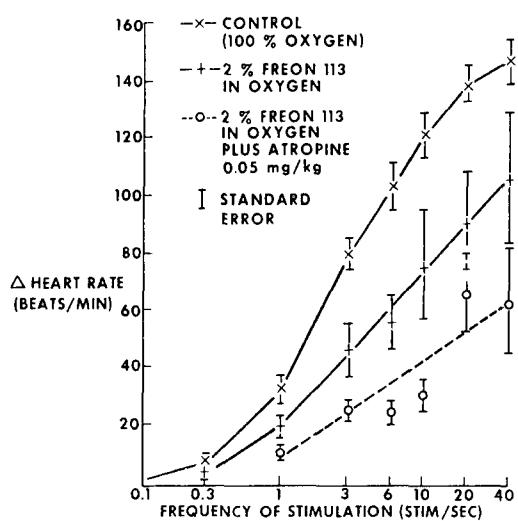


Figure 1. THE EFFECT OF 2% FREON 113 ON PREGANGLIONIC STIMULATION

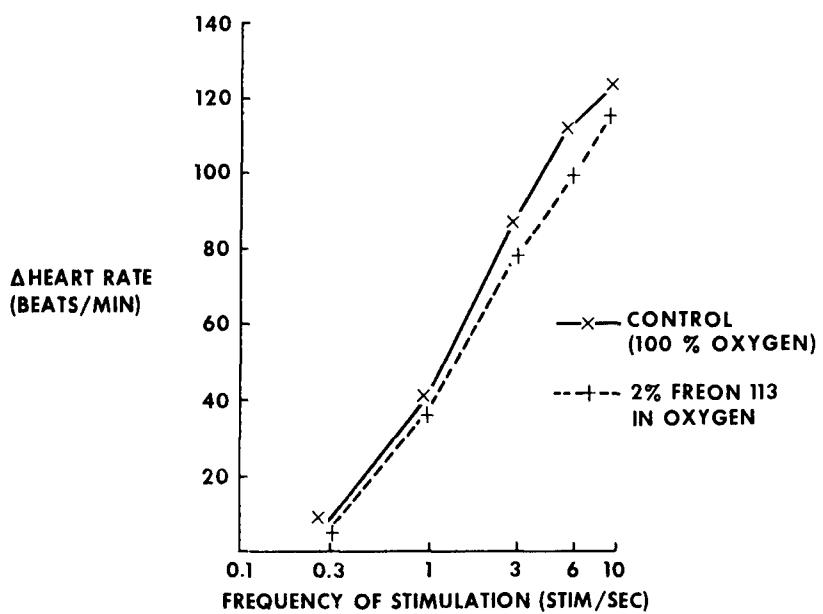


Figure 2. THE EFFECT OF 2% FREON 113 ON POSTGANGLIONIC STIMULATION

Garfield et al (1968) utilized this preparation to determine the effect of certain anesthetics on nicotinic and muscarinic transmission through autonomic ganglia. They found that halothane, among other anesthetics disrupted both classes of cholinergic, ganglionic transmission. The degree of nicotinic blockade produced by 2% halothane as demonstrated by these investigators is presented in figure 3. Although comparing data of this type between laboratories is very difficult, it does appear that the nicotinic blockade produced by 2% halothane and 2% Freon 113 are comparable.

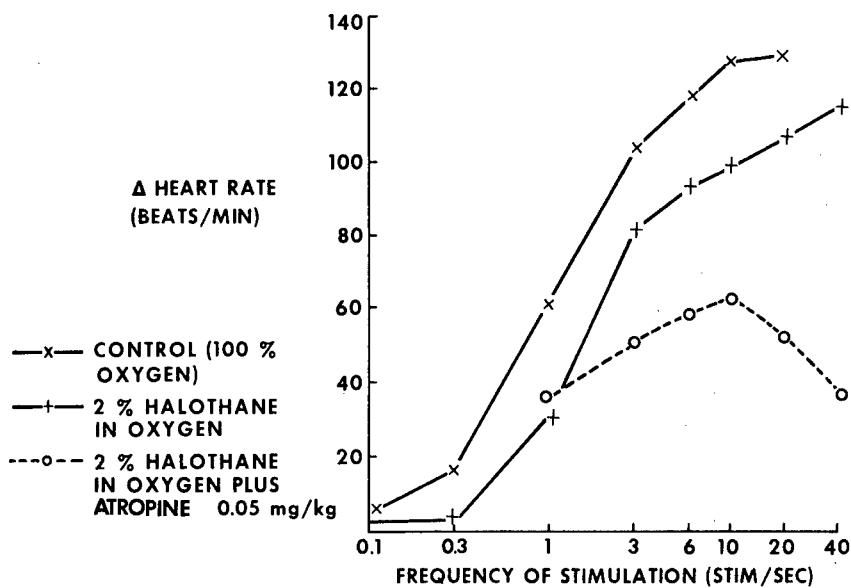


Figure 3. THE EFFECT OF 2% HALOTHANE ON PREGANGLIONIC STIMULATION

\*Taken from Garfield, J. M., Alper, M. H., Gillis, R. A. and Flacke, W. (1968). A Pharmacological Analysis of Ganglionic Actions of Some General Anesthetics. Anesthesiology 29, 79-92, and used with the author's permission.

The results of Garfield et al (1968) suggest the possibility of a relationship between the ability of certain anesthetics to antagonize both nicotinic and muscarinic transmission in sympathetic ganglia and their efficacy as anesthetics. Burn (1959) has shown that approximately 5 times the concentration of Freon 113 compared to halothane is required to produce anesthesia in mice. Our data indicate that Freon 113 and halothane in equal concentrations have approximately equal efficacy in reducing nicotinic transmission through the stellate ganglion of the spinal dog. Unanswered is the ability of Freon 113 to reduce muscarinic transmission through the ganglion, and if so, at what concentrations. It is entirely possible that anesthetic concentrations of Freon 113 would be required to antagonize muscarinic transmission. If so, this may help explain the large difference between the concentrations required to produce initial central nervous system depression and that required to produce anesthesia.

#### SUMMARY

Four monkeys, 8 dogs, 40 mice and 50 rats were exposed continuously to 2000 ppm Freon 113 in a Thomas Dome for 14 days. This exposure produced no mortalities nor adverse symptomatology. There were no significant alterations in hematological values, clinical chemistries, electroencephalographic findings, body weights, or organ to body weight ratios. The effect of 2% Freon 113 on nicotinic transmission through the stellate ganglion of the spinal dog was also evaluated. This exposure induced a reduction in nicotinic transmission comparable to 2% halothane. The effects of this compound on muscarinic ganglionic transmission were not evaluated.

#### REFERENCES

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2. Garfield, J. M., M. H. Alper, R. A. Gillis and W. Flack; "A Pharmacological Analysis of Ganglionic Actions of Some General Anesthetics"; Anes., 29, 79-92, 1968.
3. MacEwen, J. D. and E. H. Vernot; "Toxic Hazards Research Unit Annual Technical Report"; AMRL-TR-70-77, Aerospace Medical Research Laboratory, Wright-Patterson AFB, Ohio, 1970.
4. Stopps, G. J. and M. McLaughlin; "Psychophysiological Testing of Human Subjects Exposed to Solvent Vapors"; Am. Ind. Hyg. Assoc. J., 28, 43-50, 1967.

## DISCUSSION

DR. MAC EWEN: As I remember from reading reports and hearing about Freon 113 at meetings, it is essentially non-metabolized or only slightly metabolized and is excreted by the lung very rapidly after exposure. Do you have any information on the levels remaining? Did you do any breath analyses postexposure?

MAJOR CARTER (NASA, Manned Spacecraft Center): No, we didn't. We do have some data from Dr. Kaplan. He did some exposures in the dome and there is the slide. The last slide in the series, and I can't really comment on it, except that these were the data that Dr. Back gave me that he found, with 20 rats exposed. At the end of seven days, we sacrificed 5; at the end of 14 days, we sacrificed 5 more; then 24 hours post-exposure, 5 more, and then 48 hours. Notice that there is no real difference between the 7 and 14 day exposure level, except for some reason in the adrenals he gets less Freon 113 at the end of 14 days than he does at 7. I suppose if he were here he would like to comment on that, I don't know what it means. Then at the end of 24 hours post-exposure you do find some in fat, although it's dropped about 85% and at 48 hours, there is less than 1% of the original level. After 24 hours there is none in any of the other tissues that he tested.

TISSUE	EXPOSURE		POSTEXPOSURE	
	7 DAY	14 DAY	24 HOURS	48 HOURS
BRAIN ug/gm	22.73 (1.00)	22.65 (1.33)	NONE	NONE
LIVER ug/gm	15.77 (0.87)	16.40 (1.72)	NONE	NONE
HEART ug/gm	16.59 (2.56)	15.03 (2.51)	NONE	NONE
FAT ug/gm	722.48 (71.29)	659.24 (21.17)	108.45 (33.62)	5.60 (2.94)
ADRENAL ug	8.39 (2.61)	3.47 (0.34)	NONE	NONE
THYROID ug	1.09 (0.46)	0.94 (2.00)	NONE	NONE

( ) STANDARD DEVIATION

DR. BACK: The reason I can't say a lot about it is because I didn't do anything but help him harvest the tissue. But this is gas chromatography and the replication is really good between the pieces of tissue that he took. Except that he took all of his data with him to Thailand, so I can't do anything but show what we've got here. Now, after the seventh day, he just took things out of the sample bag for analysis, and I did all the cutting myself so I have to take the blame for the adrenal gland, I think that I left too much fat on the adrenal gland. At the fourteenth day, I used a head lamp and I got every bit of the fat off, they were absolutely clean and I think this is why you see the difference. If you notice that the fat does pick it up after a relatively long exposure. We did some cursory experiments at the end of an exposure for 30 minutes, and the fat does not have time to pick it up under those conditions. So it's blown off very, very quickly in the breath so that if animals are exposed for 30 minutes, 10 minutes after exposure it's all gone. This is also true with Freon 1301, but when you do exposures for 14 days, I think you could see that it is picked up pretty well and in fact 24 hours later there is still some in the fat, and 48 hours later there is still a little bit. It is all gone the next time he looks at it.

MAJOR CARTER: Before Colonel Steinberg asks his question, we also did some other exposures in connection with the autonomic ganglia work. We exposed dogs at 1% and there was no effect also exposed some to 4%. Right now I'm trying to have that data compared with 2%. We used the animals as their own controls. It is very difficult to compare four animals here with four animals over there. Our statistician at NASA says he has a method for doing this. I hope he's still there when I get back. But there doesn't really appear to be any difference at 4% compared with 2%, as far as transmission through the nicotinic portion of the autonomic ganglia is concerned.

COLONEL STEINBERG: The second question I was going to ask you: this didn't look like a complete blockade, so you would expect to get a little more blockage with increase in the dose, but one question, this is an open chest dog you stimulated?

MAJOR CARTER: Right.

COLONEL STEINBERG: Well, one thing I didn't notice, maybe this is on the slide, is after you've blocked the heart and vagotomized it in an open chest preparation you would expect that when you did do postganglionic stimulation, you should have gotten a greater response than you got in your initial controls, because you should have a sensitive preparation in terms of stimulation.

MAJOR CARTER: Right. You mean the effect of the compound should cause a potentiation of the postganglionic response?

COLONEL STEINBERG: Right.

MAJOR CARTER: Because of the sensitization of the myocardium? We didn't see this. It has been seen with Halothane. At 0.25% with Halothane, and postganglionic stimulation, you do get a larger response than you do with no Halothane. Now, at 1% you're getting enough blockade that you're essentially seeing nothing. You've potentiated your response, as far as the heart goes, and you have a small degree of ganglionic blockage but the thing is controlled when you do stimulate postganglionically. You will see a slight potentiation. Now, I think maybe we might have seen this if we had had a pure postganglionic nerve. But in only one dog did we get what we considered a pure postganglionic nerve. In one animal we had after C-6 we would get about a 30% drop in heart rate increase from stimulation of supposedly postganglionic nerve, which would indicate that there are some preganglionic fibers there. I don't know why, we're putting the electrodes the same place, supposedly that the other people were, so I have no explanation. I can't really say as to whether we were getting some potentiation there or not. Does that answer your question?

COLONEL STEINBERG: Yes. If that was an open chest since that's one of the ways used to simulate heart failure, do you think this had an effect? What did your EKG show?

MAJOR CARTER: The EKG was quite rapid. The baseline rate runs around 120 to 150. Now, you're driving it so that you get a delta heart rate of around 150, that means that you're driving the heart around 300 and I was quite surprised because I thought we would see some PVC but we didn't. And this makes one wonder what causes the PVC. One would think that you would get enough epinephrine released from the nerve, if this is contributing to the PVC's that you are supposed to see with these halogenated compounds. Now I notice in the literature, Garfield and coworkers, they didn't see any PVC's either with Halothane. So I don't know. Dr. Back, I think, has a comment.

DR. BACK: I think there are two things you've got to look at here from some work that Major Van Stee has done. One of them is that you're working against yourself because the compound probably does cause a decrease in diastolic pressures, and that the heart is not capable of pumping that hard, maybe. So, you've got that going against you, and also I think it has been shown with 1301, which probably can carry over to this compound, that you've got a change in total peripheral resistance. In this preparation you've got a 50 mm blood pressure, now Major Van Stee showed that it could mechanically increase blood pressure and hence increase cardiac irritability. In other words, mechanically he could titrate an animal in and out of fibrillation merely by giving more blood or taking away blood, hence increasing or decreasing blood pressure. So that as he dropped the blood pressure, the fibrillation spontaneously quit. Now with a 50 mm blood pressure maybe this is another compensatory thing that won't allow it to do it. What do you think about that, Major Van Stee?

MAJOR VAN STEE (Aerospace Medical Research Laboratory): I think that the hypotensive state of this animal probably provides the answer to the absence of PVC because in a similar way to cyclopropane, epinephrine induced arrhythmias and Halothane induced arrhythmias are sensitive to blood pressure, but I would be reluctant to suggest a mechanism by which this was brought on. One may mechanically alter blood pressure

by a number of ways; by expansion-reduction of the circulating blood volume; by alternately constricting and releasing the aorta, methods of this type, and control the appearance of PVC's. And since we've been working with animals which were hypotensive and perhaps uncompensated heart failure too, I would guess, they probably did have an elevated left ventricular and diastolic pressure. This is probably why you didn't see any PVC's. Circulating catecholamines decrease blood pressure threshold required to trigger arrhythmias, as in the case of 1301, but I would guess that down around 50 or 60 torr mean blood pressure that you probably would not have a high enough blood pressure to trigger arrhythmias.

MR. STEVENS (Ohio State University): Major Carter, do you attempt to antagonize any of the effect of some of these compounds?

MAJOR CARTER: Did I attempt to antagonize the blockage that I saw, I'm sorry I don't understand the question.

MR. STEVENS: Did you attempt, with other compounds, to block the effect of some of these Freons?

MAJOR CARTER: No. Only the administration of atropine to cause a further shift in the curve to demonstrate that it was a nicotinic blockade. Now, one could ask, does the compound possibly have any muscarinic blocking properties? In this prep, which is seen also with others, when Eckart first demonstrated in some autonomic ganglia that there was a muscarinic compound, atropine alone does not cause a shift in the curve. To demonstrate muscarinic blockade one must first give the C-6 and then administer the compound. If you get a further shift in the curve this would indicate muscarinic blockade. We didn't do this mainly because I didn't have time, I had to leave, Dr. Chikos had to leave. I would say that, since Halothane has no muscarinic blocking properties and any of the compounds along this line seem not to, that this compound wouldn't either. I think that the blockade that we see is pure nicotinic blockade. But I can't say for sure.

DR. JACOBSON: Are there other questions? If we exhausted Freon 113 and Major Carter, both, would you care to explore Air Quality Criteria any further?

MR. ADAMS: Dr. Hueter, can you give us your definition for total hydrocarbons? I've always been confused by this when I've seen it in the literature. What are your ideas on this?

DR. HUETER: The criteria document that has been published, I believe entitled "Reactive Hydrocarbons", was intended to respond to reactive hydrocarbons and the need for the criteria document was not intended to be a health effect need. It was intended to be a need so we could tie the package together for photochemistry. Whereby, you can control hydrocarbon, and say  $NO_X$  and thereby end up with something that also controls oxidants. You can't consider one of these without considering the other two. So, of course, as total hydrocarbons are generally measured, they're expressed as

methane. Other than having some important relation to photochemistry, I don't think they have any definitive meaning in terms of toxicology. You have to measure the individual compound if you're talking about toxicology. I don't know if I've answered your question, I tried to explain really what was done, what was the intent of being done. That was one reason why the polynuclear carcinogens were not included in that document.

DR. SCHEEL: Dr. Hueter, I think the question was intended to ascertain whether there was any differentiation between ketones and aldehydes, as opposed to flat-out hydrocarbons. As I understand the total hydrocarbon, this is an all-inclusive term which simply means all burnable carbons.

DR. HUETER: That's what I meant by saying it's an expression of hydrocarbons in terms of methane. So I'm in complete agreement with what you just said.

DR. MAC EWEN: I'd like to go one step further. I know it's not fair to ask you to comment on the paper given by Mr. Stevens earlier and expect you to answer. But in his slides he showed that the gas chromatographic separations, measurements of the total hydrocarbon and methane, methane being a direct measurement did not include the hydrocarbons. The bar graphs showed the methane portion of it to be almost 95% of the total hydrocarbons. Could you expect this to be representative of raw gasoline or refinery wastes? There would be very little left if 95% is methane.

DR. HUETER: I'm really not an atmospheric chemist or air quality man either, I would only say that it is in Los Angeles, for example, the product of primary automobile exhaust and I believe that Mr. Stevens did say that most other hydrocarbons, other than methane are reactive hydrocarbons and have input into photochemistry.

DR. MAC EWEN: The reason I comment is that methane was a direct measurement and the other total hydrocarbons were expressed as methane so that the difference could be a real difference. I'm not sure of the absolute numbers but the percentages showed very little difference.

DR. HUETER: Yes, I believe you're right, but I think that the one point he was making was that it is NAPCA's feeling that we must have a method of differentiating between methane and other hydrocarbons in order to effectively institute any kind of meaningful control procedures.

MR. TOLIVER: When they speak of total hydrocarbons are they speaking only of compounds particularly of hydrogen and carbon, and no other heteroatom, or any carbon bearing compound that's basically organic?

DR. HUETER: I can answer the question by asking a question. Since I am not a chemist, the methodology for measuring the total hydrocarbon is flame ionization, whatever that measures. Will that answer your question?

MR. TOLIVER: That was the reason for my question because your total hydrocarbon can be quite deceptive if it is measured by flame ionization because you detect the response to heteroatoms, that is other than carbon for instance, oxygen, sulphur give you a negative result and what one gets is an additive result of the entire species present. So it is possible to have other chemical species present in appreciable concentrations that will reduce significantly what you're seeing.

DR. HUETER: I think it's obvious also, that NAPCA is not happy with their present method of measuring it.

DR. SCHEEL: I'd like to say thank you for putting Mr. Toliver on the program this afternoon because I think now that if he would write up his paper and put it into the proceedings, we will finally get some agreement between gas chromatography in different laboratories. This would be a great assistance to most people, I've found, the information they've covered is so scattered that they don't bother to dig it out, put it together, and I think he did a beautiful job of putting it together for us.

MR. TOLIVER: I wanted someone to ask me a question because one finds when one finishes the paper, you never quite say exactly what you wanted to say. I did want to add this that this particular approach is sometimes called the poor man's mass spectrograph and the reason for this is that one gets a number like 468, which I had on there as ethanol and from this number one can determine that this represents ethanol. So it is a way of identifying compounds and reproducing it from laboratory to laboratory which is a most important thing.

MR. VERNOT: The problem I have found is that retention time whether relative or absolute is a function of many unknown factors.

MR. TOLIVER: Retention time?

MR. VERNOT: Retention, any measure of retention, as a means of identification of materials analyzed gas chromatographically. When you are faced with a reasonably complex mixture, say something like 25 compounds, as an old gas chromatographer you know you're probably not going to get 25 peaks, you're going to get superimposition of peaks. You're not going to get nice, easily characterized compounds, you're going to get polyfunctionality both within any one particular molecule and among the molecules themselves. When you try to use any systematically developed but empirical technique, which is what many of the index techniques are, you soon find that you're lost in a maze of complexity, because trying to run under various conditions, not only would your retention times change, but the number of peaks would change, and you don't know which peak is which when you look at the different charts. Now, it's true when you're dealing with one compound, or with two or maybe with three, this index identification is a fairly simple matter, but as soon as you get into a somewhat complex situation then the whole thing tends to fall apart and this of course is where specifically the mass spectrometer becomes quite important, even though there are other means of identification of the materials. It lets you see what is coming out of the gas chromatograph.

MR. TOLIVER: Thank you, and I'm glad you brought this up. Two problems: coincident peaks, one has to assume, of course, that the primary function of a gas chromatograph is to separate, and under optimum conditions, one can separate ideally as many compounds as possible according to the phenomena going on in the column. However, one can get coincident peaks, but what one finds instead of using one column, unless the compounds are very, very, very much alike, and we are now able as you know, to separate optically actively compounds, which means that they are simply mirror images of each other. There are certain other new areas that we have in column technology that allow us to vary, to separate most of the kinds of compounds that make coincident peaks. So I must say that if we are good gas chromatographers we can analyze 95% of the compounds we come across. Most good analytical techniques don't require that you analyze more than 95%.

MR. VERNOT: I'll keep this short. The fact is, however, that when you have a large number of peaks and you run them under different conditions, you don't know which peaks were what in the original gas chromatogram. You can't extrapolate back from one peak in one gas chromatogram to the same peak in another chromatogram.

MR. TOLIVER: Let's assume that we're talking about, let's say ten compounds. And the ten compounds all come out with ten different retention numbers, right? Now any one or more of these ten compounds have one or more peaks in them, right?

MR. VERNOT: Yes.

MR. TOLIVER: All right fine, if one runs another gas chromatograph and another column, obviously because of the intermolecular forces in there, one should not get the same kind of separation. True?

MR. VERNOT: No, but you don't know which is which.

MR. TOLIVER: But you do come up with a number, there is a possibility of having the same number as one has with almost all other instrumental approaches, you can come up with the same number. But not on the same column, and according to the number of columns you have run, you reduce the likelihood of not being able to identify the compound. Obviously there are some compounds, I'll admit readily that perhaps 5% of the compounds, we cannot separate. This is not a fault of Kovats Indices, this is a fault of gas chromatography.

MR. VERNOT: I'm not sure I can answer that. Let me say something about mass spectrometry, that is that it's true that there are problems as far as mixtures are concerned, but if the mixtures are kept, let's say to four components---

MR. TOLIVER: You said 25.

MR. VERNOT: No, I'm talking about any one gas chromatographic peak now. You have four components in any one gas chromatograph peak, it's not a difficult thing to first find out that you have four components from purely mass spectrometric information and then to analyze what those things are.

MAJOR ARNOLD (Aerospace Medical Research Laboratory): I was just going to ask Ed, how many peaks he would imagine that he is going to get out of this mass spectrograph when he injects a 25 component mixture into the mass spec?

MR. VERNOT: I'm not sure I--you mean mass spectrometric peaks, well, of course, that's how you analyze the mass spectrum. You may or you may not. It depends on how that particular compound or mixtures of compounds break down in the mass spectrometer.

MR. TOLIVER: But one of the difficult problems that we had, when we talked about this particular approach with mass spectroscopists is that they think we're attacking mass spectrometry. I'm not attacking it, I'm simply suggesting an approach that says the more positive information you have on identification, the more likely you are to identify the compound. So I simply suggested another method to identify the compound, and for those people who don't have the money to buy sophisticated and more expensive instruments, this is a way of representing the data in the open literature that can be duplicated.

DR. SCHEEL: I have had access to both these techniques and used them both and I would just simply like to comment that the gas chromatograph does have one advantage over the mass spectrometer, especially when you start talking about cyclic compounds, if they contain more than one ring. The gas chromatograph is able to separate these and give them to you in single peaks, which you can then run into your mass spectrograph. The mass spectrograph breaks down compounds and you first have to find out how they break before you can identify them. Now this particular facet of the gas chromatograph is especially valuable because we very rapidly, in biological systems, run out of straight chain compounds and run into cyclic compounds, which may be heterocyclic or homocyclic, depending upon what we are looking for. The sensitivity in the past years on the gas chromatograph has been increased tremendously. We, in addition, can use isotopic labelled compounds and calibrate specifically. So, I think there are places for both techniques and I think they both have a tremendous use, but the point that we brought out in Mr. Toliver's talk here, was that he has now taken exactly what Vernot was saying was the trouble and begun to put it down in numbers, which become identifications because you run your calibration curve with a known compound for the column. This then allows us to now compare this column with another column and another column and another column and come up with the same answer.

MR. TOLIVER: Thank you for your help, but let me correct you just this much. The numbers are run by a standard referee in London. There are about five or six in the world and the numbers that we get off the instrument are basically not run in terms of standards per se but between the homologous series. It's a small point.

DR. SLONIM (Aerospace Medical Research Laboratory): There is one thing that I didn't understand in your talk. If you had a mixture of compounds, a large mixture and suppose you did have coincidental peaks, how do you know you have more than one compound, if it only shows one peak at that particular retention number?

MR. TOLIVER: All right, let's assume that the molecular structure of the two compounds is different, but because of the hydrocarbon portion of the molecule and the functional portion of the molecule, let's make one an acetone group and the other an alcohol. You've got C = O and OH group. On that particular column they both come off at the same place. Now, as you probably know, alcohol is a little more polar than acetone, so when you put it on a little more polar column, the intermolecular forces would cause alcohol to be held longer and you should get a separation. Did I answer your question? I'm saying that if the molecular structure is relatively different that you can separate them on different columns.

DR. SLONIM: But you've had information prior to this, I mean, you already had some idea of the compounds, in fact whether there are polar or non-polar groups here. Let's assume you have a mixture and you do have a couple peaks where instead of four or five compounds as your chromatogram mixture, you may be dealing with about ten compounds. My question is would it be false security assuming that that's all you had, if in fact you do have multiple peaks as a result of the mixture, which sort of supports what Ed Vernoit was saying as to whether or not, using your gas chromatograph, unless you knew exactly how much you had in there, you wouldn't even know to go to another column to separate them?

MR. TOLIVER: One normally uses more than one column for this kind of identification. The original work, you will notice, reported a polar and a non-polar column. But always a minimum of two columns of different separation characteristics. Of course, one can go through as many columns as one likes to identify beyond a doubt what the compound is.

DR. SLONIM: You feel that given one retention number from one peak and by using more than one column in time----

MR. TOLIVER: Let's see if I can do this thing on a particular column, let's take Apiezon L since it's probably the most worked column. It is possible to take the number 468 and have more than one compound that has that number, but the likelihood of that compound on another column, those five compounds from another column having the same number are reduced. And the more columns one uses, the more one reduces the probability of that particular number falling at that same spot. That's the same kind of thing that one does in infrared, the same kind of thing that one does in most analytical methods. No difference, absolutely none.

DR. JACOBSON: Are you interested in pursuing this further, Mr. Vernot?

MR. VERNOT: If I gave the impression that I wanted to substitute mass spectrometry for gas chromatography, I certainly didn't mean to do that. Modern analytical chemists, dealing with materials of any vapor pressure at all, cannot do without gas chromatography and we can't do without it here. I'm merely saying that there are presently so many techniques available for the marriage of gas chromatography and mass spectrometry, that if a mass spectrometer is available, one can get this kind of information as far as identification of molecules of interest, perhaps more unambiguously than by Mr. Toliver's method.

DR. MAC EWEN: I have a question I would like to ask Dr. Ingram or rather two questions. The first one is how do you get an even blood smear for use in the cell scan GLOPR?

DR. INGRAM: Well, we have developed a new method for producing smears which is remarkably good, this is not really new, it's only new for smears. It is adapted directly from the technique used for coating small optical parts with photoresist. If I could draw a little picture, I can explain it very quickly. This is a spinner technique that I was using. We use a spinner made by Pratt Engineering Company in Chatham, Pennsylvania. They make it for industrial use and any other purpose. It has a rotor spinner, slide, and once in position a cover glass. It's held on by a powerful suction to the spinner and there is a safety catch that goes on when it spins. It sounds like an outlandish method, but using blood which has been treated with EDTA to prevent coagulation and clumping, the cover glass is placed down here. Then we flood it with the blood which has been mixed so that there is a fairly uniform suspension of cells. You can actually see the top of the meniscus over the whole cover glass. The cover glass rotates and it is designed with a clutch mechanism which can be used so that you reach full speed in about 30 milliseconds; or you can use a slower speed of one second. After that it really doesn't seem to make much difference if you slow it down further. There is a speed regulator. Push the button and it spins rapidly, the blood goes flying off in all directions and you end up with a perfectly beautiful, absolutely uniform smear of blood cells, bone marrow, blood platelet suspensions--what have you.

It's really remarkable, we did a lot of work to document this. We did a lot of cover glass smears, differentials, and cell counts with Professor Garret. The cell distribution over the smear is absolutely random. There's no collection of large cells. At the extreme edge there's a very, very thin rim which is different, but that's negligible.

The thing that's most important about it is that there is almost no disruption of the cells that you get with any other method of making smears. This makes the very best cover glass smears I have seen. If you pick them out very critically and then force yourself to count how many cells are broken or disrupted, there are all kinds of variables that can be recognized. So if you count them, they'll turn out to be about 12 or 15 percent of all the leukocytes where the cell membranes are severely disrupted.

The remarkable thing there is absolutely no sign of sagging and the cells are uniformly flattened. The reason that they're so uniform is that when you put the blood on the slide the leukocytes and platelets stay precisely where they land and the red cells spin off. Now we have another interesting point concerning the matter of spinning speed. If one spins at speeds of about 5000 or 7000 rpm at the end, there will be areas where there is a blood clot and blood cells are perfectly beautiful in morphology. There is no displacement of intracellular contents at all. But, red cells come smashing up against them and against the blood platelets, and get deformed. They are often elliptical in shape and the central area may be displaced. If you just look at these you think they're beautiful for leukocytes but not good for red cells. You can get around this by spinning at 4000 rpm and putting a lid on them. Then you get a nice round red cell contour. But, my own feeling is that this effect is extremely important. This is a very easy way of studying red cell deformability. When you get an abnormal blood, you can see that the degree of deformability of the red cells is extremely variable from normal to abnormal specimens and among abnormal specimens. I think this might turn out to be very important.

DR. JACOBSON: Thank you, Dr. Ingram. Dr. MacEwen, did you have an additional question?

DR. MAC EWEN: Yes, I did. The second question I had, and I'm not sure I completely understood you this morning about the cell scan or the cell scan GLOPR. I got the impression that this was sort of a universal do-it-all and that if you put your blood smear on it you could obtain a red blood count, white blood count, a platelet count, a differential and perhaps even a reticulocyte count from the computer output. Is that correct?

DR. INGRAM: No, that is not quite correct. It is a system for classifying cells and getting quantitative measurements of certain morphological characteristics. It's really an image analyzer system that's concerned with the morphological characteristics of cell images.

DR. MAC EWEN: But it can differentiate between all of these cell forms?

DR. INGRAM: Yes.

DR. BEARD: I was just a little concerned about this spinning device you described as a possible way of making an aerosol of hepatitis virus.

DR. INGRAM: This is why it is now made with a cover. We actually made a cover for all of ours with a piece of safety glass over the top, but you can use it in a hood or with a dome over it, if you want to make deformability studies, and still be quite safe. We were aware of this hazard which is a very good point, as soon as we got the additional work done and published the paper on the method. The manufacturer then brought out the modified instrument with the cover on it. We could see this problem arising right away.

TOXICOLOGY OF PROPELLANTS AND OTHER MILITARY CHEMICALS

Chairman

Dr. Kenneth C. Back  
Toxic Hazards Division  
Aerospace Medical Research  
Laboratory  
Wright-Patterson Air Force Base, Ohio

## ACUTE TOXICITY OF OXYGEN DIFLUORIDE

Harvey V. Davis, Ph.D.

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

### INTRODUCTION

Because of the potential use of oxygen difluoride ( $OF_2$ ) being used as an oxidizing fuel in the missile industry, it became necessary to define and characterize the hazards associated with the handling of this compound. The gas has been characterized as a strong oxidizing agent, highly toxic, with properties similar to elemental fluorine. It is colorless at atmospheric temperature and pressure, condensing to a pale yellow liquid at -145 C (American Industrial Hygiene Association, 1967). It is stable in dry air and decomposes to any appreciable extent only at elevated temperatures (Allied Chemical Corporation, 1962).

The inhalation toxicity of  $OF_2$  was first reported by LaBelle who demonstrated the highly toxic nature of the compound by using several species of animals (LaBelle et al, 1945). The assessment of  $OF_2$  toxicity in this study was made by exposing four species of animals (monkeys, dogs, rats and mice) to various concentrations of the gas for 15 and 60 minutes.

### METHODS

The  $OF_2$  used was a commercial grade purchased from the Allied Chemical Corporation. Assay data indicated 98% purity, most of the impurities being oxygen with trace amounts of carbon dioxide ( $CO_2$ ) and carbon tetrafluoride ( $CF_4$ ).

The gas was diluted with dry nitrogen in the Dilution Facility to give a concentration of approximately 1%  $OF_2$ . The large cylinders containing the  $OF_2$ -nitrogen mixture were pressurized at 1000 pounds and analyzed for the precise  $OF_2$  concentration, after which the diluted gas was delivered to the Toxicology Laboratory. This procedure was developed to minimize the hazards resulting from a possible accidental exposure.

The gas in diluted form was used for the toxicity studies. All exposures were made in the Longley exposure chamber shown in figure 1. The exhaust from the chamber was passed through a water scrubber, with caustic added, to remove  $OF_2$  from the stack effluent. The MSA Billionaire was the analytical instrument used to monitor the concentration of the gas in the exposure chamber.

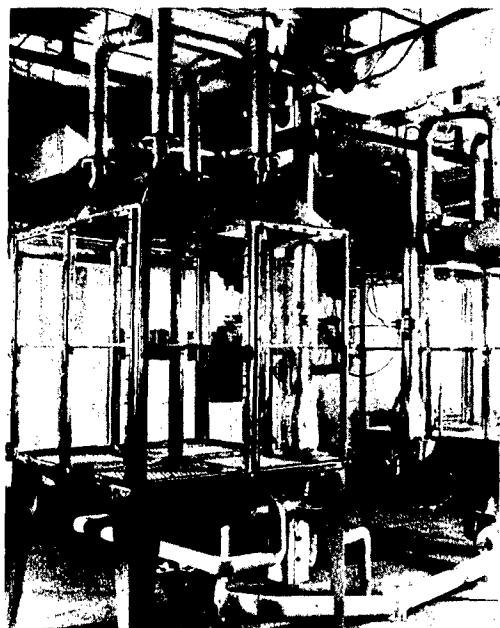


Figure 1. LONGLEY EXPOSURE CHAMBER

The exposure consisted of four species of animals: Beagle dogs and Rhesus monkeys of both sexes; male Wistar rats, mean weight 250 grams; male ICR mice, mean weight 35 grams. The usual number of animals in each exposure consisted of 4 monkeys, 4 dogs, 10 rats and 15 mice.

## RESULTS

The animals were exposed to various concentrations of the gas for 15 and 60 minute time periods. The evaluation of  $OF_2$  toxicity was made by measuring several physiological and biochemical parameters.

1. Symptomatology was observed during exposure and up to 14 days postexposure.
2. Mortality response was recorded for the same time period.
3. Biochemical and hematological tests were made on a selected number of animals.

4. Gross and histopathology examinations were performed on tissues from animals exposed to both lethal and sublethal concentrations of the gas.
5. The phenomenon of tolerance induction in rodents was examined.

### Symptomatology

Outward signs of  $OF_2$  toxicity manifested itself in various forms. During the exposure, respiratory distress was the most common symptom seen in rodents, characterized by a rapid, shallow breathing pattern. Gastrointestinal and upper respiratory tract irritation was seen in both monkeys and dogs, although less severe in monkeys.

Survivors of each species exhibited various forms of dyspnea for several days postexposure. One of the most surprising findings was the lack of skin irritation even in animals exposed to lethal concentrations of the gas. A summary of symptoms observed during the exposure is listed below.

TABLE I  
SYMPTOMATOLOGY DURING EXPOSURE

<u>Species</u>	<u>Symptoms</u>
Rats and Mice	Tachypnea Muscular Weakness
Dogs and Monkeys	Gagging Lacrimation Salivating Muscular Weakness Dyspnea Vomiting Tetany

### Mortality Response

Animal mortality was recorded for both the 15 and 60 minute time periods. Based on the CT (concentration x time) there was a linear response over the time range studied for each of the species. The following CT values were obtained:

Monkeys	at 60 minutes, 15 minutes,	26 108	ppm ppm	(CT = 1560) (CT = 1620)
Dogs	at 60 minutes, 15 minutes,	26 90	ppm ppm	(CT = 1560) (CT = 1350)
Rats	at 60 minutes, 15 minutes,	2.6 12.7	ppm ppm	(CT = 156) (CT = 191)
Mice	at 60 minutes, 15 minutes,	1.5 7.5	ppm ppm	(CT = 90) (CT = 113)

The most significant findings were the differences in mortality response between rodents and the large animal species. This response was an order of a magnitude different and is in agreement with published rodent toxicity data (Cianko, 1961; Dost et al, 1968; Lester and Adams, 1965). There was no available information, however, on the susceptibility of monkeys and dogs to OF<sub>2</sub> intoxication. Monkeys and dogs were found to be less sensitive to the toxic effects of the gas. Based on data obtained from accidental human exposures, it appears that man responds to OF<sub>2</sub> in a manner similar to that observed in monkeys and dogs (MacEwen and Vernot, 1969). The mortality response to the inhaled gas is summarized in tables II, III, IV and V.

TABLE II  
SIXTY MINUTE MORTALITY RESPONSE  
DOGS AND MONKEYS

<u>Species</u>	<u>No. Exposed</u>	<u>Conc. (ppm)</u>	<u>Mortality Ratios</u>
Monkeys	4	16.0	0/4
Monkeys	4	21.0	1/4
Monkeys	4	32.0	3/4
Dogs	4	8.2	0/4
Dogs	4	16.0	2/4
Dogs	4	21.0	1/4
Dogs	4	32.0	4/4
		Monkeys LC <sub>50</sub> 26.0 ppm	
		Dogs LC <sub>50</sub> 26.0 ppm	

TABLE III  
SIXTY MINUTE MORTALITY RESPONSE  
RATS AND MICE

<u>Species</u>	<u>No. Exposed</u>	<u>Conc. (ppm)</u>	<u>Mortality Ratios</u>
Rats	10	2.2	0/10
Rats	10	2.7	7/10
Rats	15	3.0	14/15
Rats	10	4.0	10/10
Mice	15	1.0	5/15
Mice	15	2.2	8/15
Mice	15	4.2	15/15
		Rats    LC <sub>50</sub> 2.6 ppm	
		Mice    LC <sub>50</sub> 1.5 ppm	

TABLE IV  
FIFTEEN MINUTE MORTALITY RESPONSE  
DOGS AND MONKEYS

<u>Species</u>	<u>No. Exposed</u>	<u>Conc. (ppm)</u>	<u>Mortality Ratios</u>
Monkeys	4	60	0/4
Monkeys	4	100	2/4
Monkeys	4	120	2/4
Monkeys	4	140	4/4
Dogs	4	60	0/4
Dogs	4	80	1/4
Dogs	4	100	3/4
		Monkeys    LC <sub>50</sub> 108 ppm	
		Dogs    LC <sub>50</sub> 90 ppm	

TABLE V  
FIFTEEN MINUTE MORTALITY RESPONSE  
RATS AND MICE

<u>Species</u>	<u>No. Exposed</u>	<u>Conc. (ppm)</u>	<u>Mortality Ratios</u>
Rats	10	16.5	9/10
Rats	10	15.2	8/10
Rats	10	13.8	9/10
Rats	10	11.9	1/10
Rats	10	11.0	3/10
Rats	10	10.4	1/10
Rats	10	9.5	0/10
Mice	15	16.5	14/15
Mice	15	15.2	12/15
Mice	15	11.9	15/15
Mice	15	11.0	8/15
Mice	15	9.5	12/15
Mice	15	8.5	4/15
Mice	15	7.5	8/15
Mice	15	5.8	1/15
Mice	15	4.5	8/15
		Rats LC <sub>50</sub> 12.7 ppm	
		Mice LC <sub>50</sub> 7.5 ppm	

#### Clinical and Biochemical Tests

Clinical and biochemical tests were performed on selected numbers of dogs and monkeys exposed to various concentrations of the gas. Tests were made immediately after exposure and at various intervals up to 14 days postexposure.

The blood constituents, uric acid, urea, and creatinine were not significantly different from those seen in control animals. This auremic condition was taken as an indication that no functional damage had occurred in renal tissue at any of the time periods tested. There were no significant changes in either serum alkaline phosphatase or glutamic oxaloacetic transaminase (SGOT). Blood glucose was normal and there were no changes seen in the extracellular electrolyte composition.

A study was made to determine the effect of the gas on the blood clotting mechanism in dogs. Tests were made immediately after, twenty-four hours and seven days post-exposure. There were no demonstrable differences between control and exposed animals as indicated by normal prothrombin times in all animals tested.

Pathology

Macroscopic changes resulting from the exposure to gaseous oxygen difluoride consisted chiefly of pulmonary damage in all species. At lethal concentrations, massive lung edema and hemorrhage with liver, spleen and kidney congestion were common observations. At sublethal concentrations there were slight to moderate degrees of lung congestion and edema.

Tolerance Induction

One of the last approaches undertaken to study the pharmacological properties of  $OF_2$  was to investigate the phenomenon of tolerance induction. Tolerance is defined as the ability to endure or resist the toxic action of a chemical. This phenomenon has been observed in animal exposures to ozone, nitrogen dioxide, and other compounds classified as respiratory irritants (Fairchild, 1967; Matzen, 1957; Stokinger and Scheel, 1962).

Our investigation was limited to the occurrence and duration of tolerance in rodents, and the characterization of the induction concentration of the gas required to produce tolerance. Mortality response was the only criterion used to determine tolerance.

A group of mice was exposed to induction concentrations of 1.0, 0.50 and 0.25 ppm of  $OF_2$  for 60 minutes. The preexposed groups along with a naive group (control group) were reexposed to multilethal concentrations of the gas for sixty minutes. Tolerance was measured at various periods up to 24 days postexposure. There was no significant tolerance produced, as measured by mortality response, in mice exposed to the induction concentrations of 0.50 and 0.25 ppm of the gas. The group exposed to 1.0 ppm (see table VI) developed tolerance within 24 hours, maximized at 8 days and was still effective 24 days after the initial exposure. This observation seems to indicate that tolerance can be produced in mice when the induction concentration is near the lethal effect level.

TABLE VI  
INDUCTION OF  $OF_2$  TOLERANCE IN MICE BY PREEEXPOSURE TO 1 PPM

<u>Group</u>	<u>Conc. (ppm)</u>	<u>Post Treatment Time</u>	<u>% Mortality</u>
Naive	3.45	24 Hours	100
Preexposed			60
Naive	4.25	8 Days	100
Preexposed			10
Naive	3.50	24 Days	100
Preexposed			50

## SUMMARY

The acute effects of  $OF_2$  inhalation were shown mainly to be respiratory in nature. Tachypnea was the most prominent toxic sign observed in rodents. Upper respiratory and gastrointestinal tract irritations were observed in dogs and monkeys.

The mortality response demonstrated a significant difference in the susceptibility of the various species to the toxic effects of the gas. Rats and mice were found to be much more susceptible than monkeys or dogs. A summary of the mortality response is listed in table VII.

TABLE VII  
SUMMARY OF  $OF_2$  DOSE RESPONSE AND  $LC_{50}$  VALUES OF ANIMALS

<u>Species</u>	<u>LC<sub>50</sub> Values, ppm</u>		<u>CT Dose, ppm-min</u>	
	<u>60 Minutes</u>	<u>15 Minutes</u>	<u>60 Minutes</u>	<u>15 Minutes</u>
Monkeys	26	108	1560	1620
Dogs	26	90	1560	1350
Rats	2.6	12.7	156	191
Mice	1.5	7.5	90	113

The most characteristic macroscopic changes were lung edema and hemorrhage. At lethal concentrations, congestion of liver, spleen, and kidney were observed.

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## DISCUSSION

DR. SCHEEL (Laboratory of Toxicology and Pathology, USPHS): Harvey, did you run succinic dehydrogenase on kidney tissue?

DR. DAVIS (SysteMed Corporation): No, we did not.

DR. BACK: I have one question. Do you have any idea about the reason for the tolerance? Increased uptake following the first exposure, or what?

DR. DAVIS: No, I think this is what can be considered a general stress phenomenon. Because it appears that, and I think Dr. Scheel can confirm this, there are about four or five mechanisms of tolerance production which have been noted. So we don't know what it is. I think it's just a general stress phenomenon, and don't ask me what stress is.

DR. DOST: If I may engage in a flight of fancy, we have suggested that the mechanism of  $OF_2$  toxicity is based on the movement of  $OF_2$  as an intact molecule into the pulmonary cell where it reacts with intracellular redox components and then results in the metabolic death of the cell with subsequent functional loss of the cell later, because the cell primarily functions as a diffusion barrier and its metabolic activity is not absolutely essential to gas transport. Now I'm wondering whether a low concentration of  $OF_2$  may be engaging in this type of reaction and resulting in increased synthesis of components that are being destroyed at high concentrations.

DR. DAVIS: This is a possibility and John Mountain has engaged in this type of research with ozone as you know.

DR. LEON: We've been playing around with this increased tolerance using animals exposed to oxygen, and George Kidd at Johnsville has been playing around with it for a number of years. Our feeling is (and I think I would be interested to find out if the same might apply with this compound since it's an oxidizing agent) that the thing that happens with these oxidizing agents is that first you get the development of pulmonary edema. If the pulmonary edema is sublethal, our histologic observations and those of George Kidd have been that it's primarily a perivascular pulmonary edema, and it's our feeling that perhaps residual or vestigial lymphatic drainage channels that were open during the fetal and postnatal period of the animal's life are reopened so that the increased tolerance is brought about by an increased capacity to remove the edematous fluid. Kidd, of course, has shown that if he exposes animals to sublethal concentrations

of oxygen at about 500 torr, he can then expose them to one atmosphere of pure oxygen and there is an increased tolerance and the histologic observation of the lungs of these animals show an opening of the perivascular, supposedly lymph, channels.

DR. DOST: One important aspect of this is the time factor. That is, the animals that are lethally intoxicated with  $OF_2$ , let's say at a minimum lethal dose, would probably not die until anywhere from 20 to 40 hours after exposure and there would be really no discernible pathology in these animals for many hours after the exposure. Such an animal examined, at 4 or 6 hours, may have a very minor amount of excess water in cells but other than this they're essentially normal in appearance and they can't be saved. This has been our experience and the experience of some of the very early investigators and I understand this is essentially what you have seen too, isn't it, Harvey?

DR. DAVIS: That's correct, yes. Ozone has been used as a model compound in this tolerance work and in nonedemic concentrations there was some tolerance produced as measured by water content. I think this was done down at Dr. Scheel's laboratory so you can get tolerance without producing edema--we've gotten it with ozone.

DR. SCHEEL: In regard to  $OF_2$ , I think we should keep in mind that there are two possibilities here for basic toxicity. The oxidizing action of  $OF_2$  can produce acute injury. But the kind of description that you give here of a 24 hour latency in deaths would fit better the fluoride toxicity that can take place if this hydrolyzes in the tissue and this forms a specific block in respiratory enzymes and I think that possibly some work on urine and fluoride excretion in the kidney with succinic dehydrogenase might explain part of the mechanism here.

DR. DOST: The problem with  $OF_2$  is that in a lethal exposure the animal will probably not contact, let alone absorb, more than about 25 micrograms of fluoride ion in the whole exposure and when this is spread out through the animal it is pretty hard to conceive a fluoride inhibition which demands concentrations of anywhere up to maybe  $10^{-3}$  M.

DR. BACK: Also, it's difficult to find out how much fluoride you've got under those circumstances. It is so small that you can't analyze it by classical techniques, and if you use radiotagged compounds you have to do it within an hour or so or you've lost it. I think the half-life is 28 minutes or some such thing as that. So you've got analytical problems when you start working with fluoride at real low concentrations like this.

DR. HODGE: You spoke of the apparent similarity of man to the larger animals in the toxic response. Has there been, is there evidence available of the application of the present TLV in some industrial situation and has there been a record of exposures and recovery or otherwise? Do we have extensive or any body of information?

DR. DAVIS: I don't think it is very extensive but Dr. MacEwen might want to give you his experience with that.

DR. MAC EWEN: There have been a few accidental exposures in the manufacturing process. They have been undocumented concentrations, they have smelled it, the man gets out and he has some pulmonary problems for a week or so. They have been hospitalized, given supportive therapy, and survived. About a year and a half ago, there was a rather severe exposure of a graduate student at a nearby university and Mr. Vernot and I went up there to investigate the accident. The man was trying to characterize the physicochemical constants of this compound. He was preparing to transfer it from a tank to a glass bottle and through it back in the analytical system. He purged his system, or cleaned his system with benzene beforehand, and apparently had saturated the tygon tubing that he was pulling the  $OF_2$  through and the system blew up. When it blew apart, the tank was unsupported and fell on the floor, bent the valve, so he couldn't shut it off. He ran over to the workbench and grabbed a wrench and got down on his knees over the tank and shut it off. The tank, however, was empty by that time. It was a one pound tank of which only about 300 cc had already been used over the year or so that they had been working with it. Making some estimates of the area where he was working, it is probable that the concentration to which he was exposed was at least 1000 parts per million for about two or three minutes before he left the immediate area. It polluted the whole laboratory building, the smell passed throughout the building and everybody else began to get symptoms too. But mostly, I think, they were sympathetic symptoms because by the time they began to get the symptoms they had heard it was toxic. The man was taken immediately to the hospital, or he hurried to the hospital, because he began to hurt and he was in the hospital for about a week and a half. He began to show improvement about the third day, the edema began to resolve. He was not given much therapy except aerosolization with alcohol, and oxygen by mask when he had trouble breathing. He did survive at that concentration and that kind of puzzled us and that's one of the reasons we went to investigate it because of the acute toxicity data on the rodent that we had read before. From Dr. Hodge's groups' work at Rochester back in the forties and the work of Dr. Scheel we couldn't explain how he could survive this kind of concentration. I think you can see from this data that the dog and monkey have an order of magnitude difference in their  $LC_{50}$  from the rodents. I think if you extrapolated that line to a two minute period, which really isn't fair because when you get down to short times like that you aren't even sure you're getting complete absorption, or complete pickup of gas in the lung, but if you extrapolated that line down to about two minutes you would find that it's somewhere around 1000 parts per million and that may explain the man's survival. Now  $OF_2$  stinks, it's been referred to as an odorless gas, but I've smelled it and it smells like garlic to me. That's the best way I can define it.

DR. BACK: This is documented for those who want a copy of what he just said, in last year's report on the THRU in 1969 on the Toxic Hazards Research Unit.

## CHRONIC EXPOSURE TO LOW CONCENTRATIONS OF MONOMETHYLHYDRAZINE

Charles C. Haun

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

The increased use of MMH as a rocket fuel suggested the need for reevaluation of the current threshold limit value of 0.2 ppm established by the ACGIH by analogy with hydrazine and unsymmetrical dimethylhydrazine. Previously reported results of acute and emergency exposure limit studies (Haun, 1968; MacEwen, 1969) performed in our laboratory provided the basis for the selection of appropriate dose levels for use in repeated inhalation studies. These tests were undertaken to determine the biological response of 4 animal species to repeated daily exposures to 2 and 5 ppm MMH for a 6-month period. Exposures were conducted on a 6 hour/day, 5 day/week basis covering a 26-week period.

Both experimental groups as well as the control set of animals consisted initially of 8 beagle dogs, 4 rhesus monkeys, 50 Wistar rats and 40 ICR mice. All animals were female except for male rats.

The Thomas Domes were operated at 725 mm Hg pressure (normal ambient pressure is 740 mm Hg) to avoid leakage of MMH, with nominal air flows of 40 cfm. Continuous monitoring of MMH concentrations was performed with an Auto Analyzer (Geiger, 1967).

Of the various parameters selected to measure the chronic toxicity of MMH, a significant number did show positive indications of toxic stress. Furthermore, in many cases, the effects were clearly dose related.

### Signs of Toxicity

Prominence of nictitating membranes of a number of dogs exposed to the 5 ppm MMH level was observed as early as 2 weeks after the initiation of this study. This effect continued throughout the duration of the study. It appeared to be minimal and in some cases absent following weekends of no exposure, but increased in severity and maximized following 4 or 5 daily 6-hour exposures. The mechanism of this effect is not clear. The animals were decidedly photophobic. Since MMH is a known irritant,

probably the simplest explanation is varying degrees of conjunctivitis. In contrast, no signs of ocular effects were observed in the case of the dogs exposed to 2 ppm MMH.

Mice exposed to 5 ppm MMH showed some signs of stress. Fur coats were rough and yellowed and the mice appeared to be lethargic from time to time. Monkeys and rats showed no observable physical signs of toxic effect.

### Mortality

Deaths occurred only in mice. Nine, six and one deaths were recorded, respectively, for the 5 ppm MMH exposed, the 2 ppm MMH exposed, and control groups. In the case of the 5 ppm MMH exposure group, seven additional mice died of accidental causes and are not included in the mortality figures. Thus, the corresponding adjusted mortality percentages are 27% for the 5 ppm MMH exposure group, 15% for the 2 ppm MMH exposure group, and 2.5% in the control group, showing a dose related effect.

### Rat Body Weights

The growth rates of the 3 rat groups are shown in figure 1. These measurements, made at biweekly intervals, illustrate the definite dose dependent effects seen in rodents from chronic exposure to MMH. The 5 ppm MMH exposed groups of rats exhibited statistically significant differences from the control group after 2 weeks exposure and at every interval thereafter. The 2 ppm exposed group of rats, while apparently growing more slowly by the second week, were statistically different (at the 0.01 significance level) by the tenth week of exposure and remained significantly depressed throughout the duration of the experiment. The weight differences between the groups is most pronounced at the conclusion - 26 weeks. There is approximately a 70 gram difference between the mean values of the 5 ppm exposed and the control, while there is approximately a 35 gram difference between the 2 ppm weight and the control. The noticeable upward excursions of values for the 2 ppm exposed and control animals recorded at the 2-week interval were caused by erroneous calibration of the weighing system (MacEwen, 1970).

### Clinical Laboratory Results

A routine battery of clinical laboratory tests was performed on blood samples taken from all large animals prior to the initiation of the experiment in order to establish baselines, then on a biweekly schedule thereafter. The group mean values for hematocrit, hemoglobin, red blood cell and reticulocyte values for all groups of exposed and control monkeys and dogs are graphically presented in figures 2 to 9. The hemolytic effects of MMH inhalation were most noticeable in dogs at both the 5 and 2 ppm exposure levels.

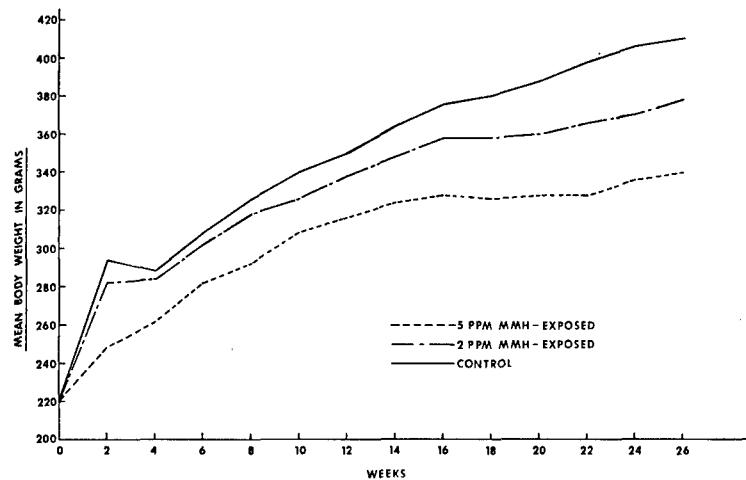


Figure 1. BODY WEIGHT GAIN IN RATS.

Figure 2 shows the effect of exposure on dog hematocrit values. The top unbroken line is the control. Values fluctuated very little from beginning to end. In contrast, sharp declines are noticeable in HCT values for the 5 and 2 ppm exposed dogs. Maximum declines occurred at 2 weeks for the 5 ppm and at 4 weeks for the 2 ppm exposed dogs. These declines were approximately 35 and 20%, respectively. After the first few weeks, HCT values appeared fairly stable until about 12 weeks, then a gradual decline was noticeable in the values for the 5 ppm exposed dogs. This continued until conclusion of the study. The overall net reduction was about 20% for the 5 ppm, and 15% for the 2 ppm HCT values. Two dogs from each exposure group were retained postexposure to study reversibility. It can be seen that HCT values surpassed pre-exposure levels, and at 2 and 4 weeks postexposure overcompensation (polycythemia) was clearly indicated.

Figure 3 is a graph of dog hemoglobin values. Maximum depressions occurred at 2 and 4 weeks. The effect of MMH on HGB was obviously more pronounced for the 5 ppm exposed group. Fifty and 20% reductions occurred at 2 and 4 weeks for the 5 and 2 ppm groups, respectively. Overcompensation was noticeable at 2 and 4 weeks postexposure.

Figure 4 is a graph of dog red blood cell values. The pattern of response is very similar to that seen in the two previous figures. The dose related effects are obvious.

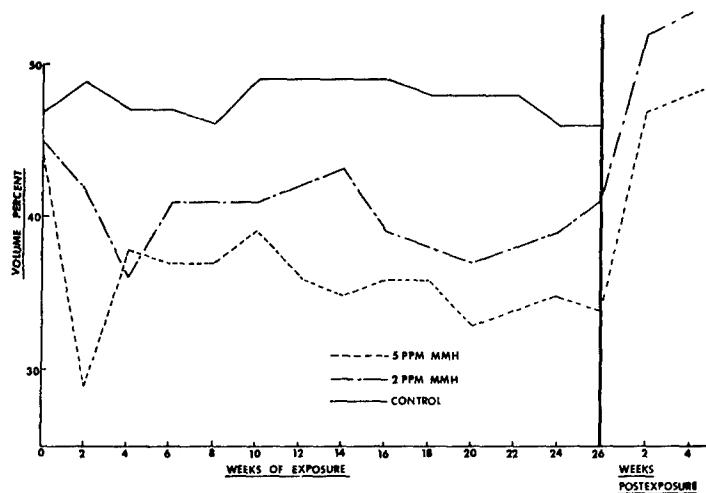


Figure 2. EFFECT OF MMH EXPOSURE ON HEMATOCRIT IN DOGS.

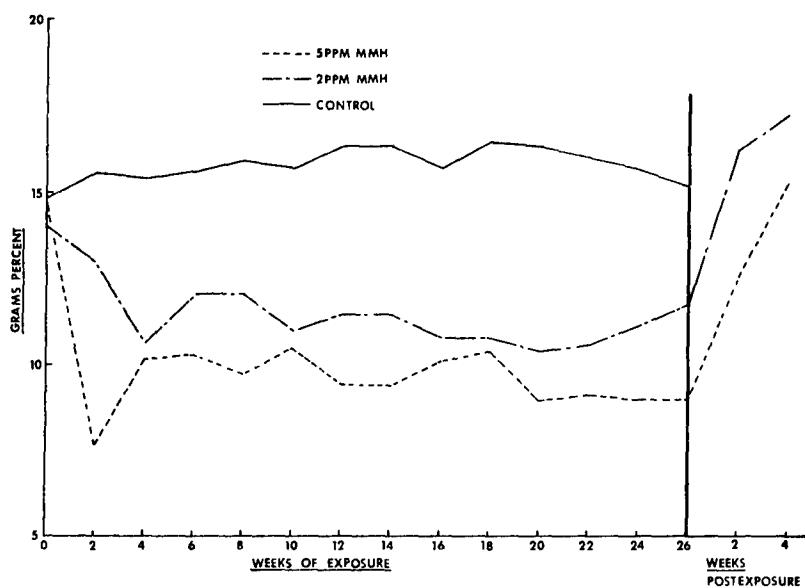


Figure 3. EFFECT OF MMH EXPOSURE ON HEMOGLOBIN IN DOGS.

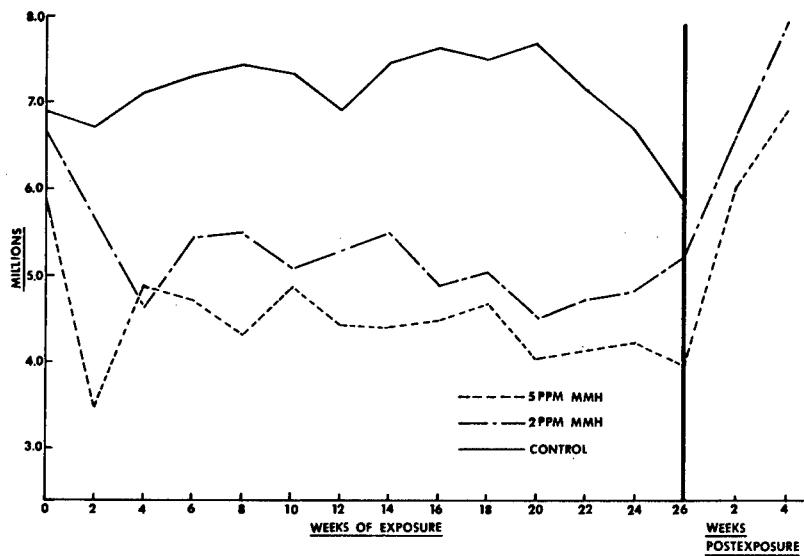


Figure 4. EFFECT OF MMH EXPOSURE ON RED BLOOD CELLS IN DOGS.

Figure 5 shows reticulocyte production in exposed animals compared with control. It can be seen that compensation for RBC destruction began within the first few weeks of exposure and accelerated in the case of the 5 ppm exposed group where some stabilization appeared to occur at 10 weeks. Unexplained were the high reticulocyte counts for the 5 ppm group at 24 and 26 weeks. However, reticulocyte values for both exposure groups declined rapidly and were essentially normal by 2 weeks postexposure.

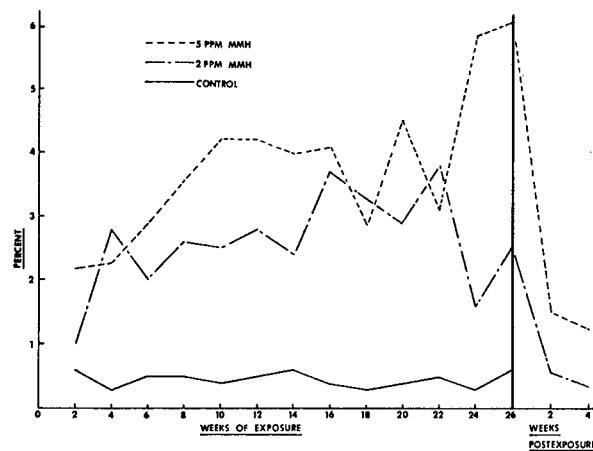


Figure 5. EFFECT OF MMH EXPOSURE ON RETICULOCYTES IN DOGS.

Statistical analysis of the biweekly data accumulated for 2 through 26 weeks shows, with few exceptions, that hematology values obtained for the 5 and 2 ppm exposed dogs were significantly different from control at the 0.01 significance level.

The hematological measurements (figures 6-9) of blood taken from monkeys on the same biweekly schedule showed this species to be less sensitive than dogs to the hemolytic effects induced by repeated exposure to MMH.

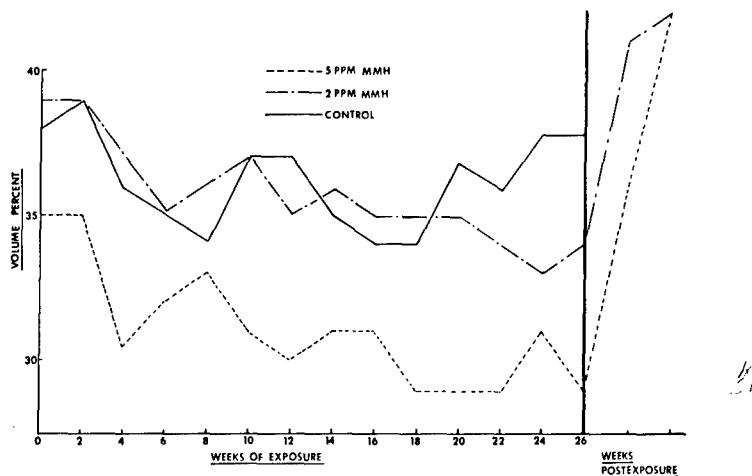


Figure 6. EFFECT OF MMH EXPOSURE ON HEMATOCRIT IN MONKEYS.

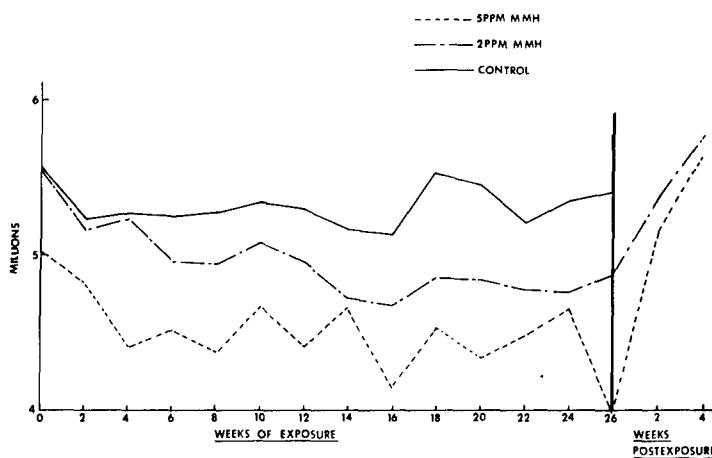


Figure 7. EFFECT OF MMH EXPOSURE ON RED BLOOD CELLS IN MONKEYS.

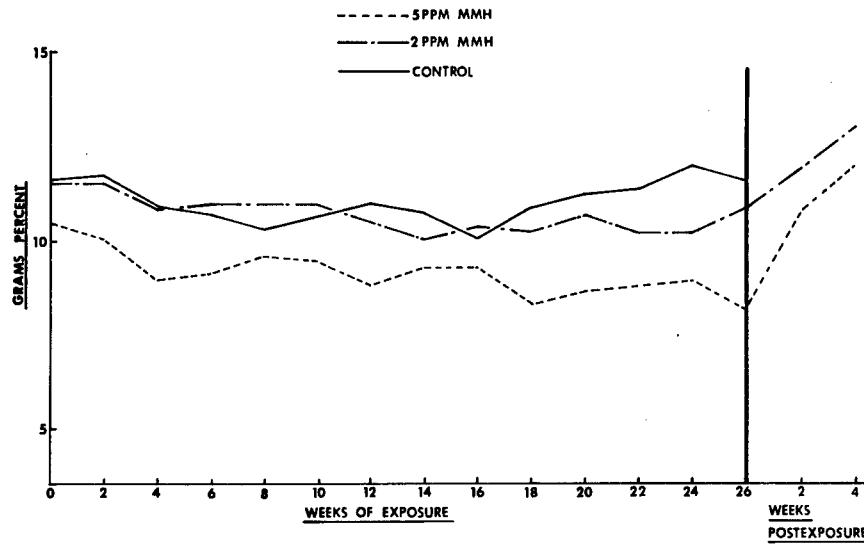


Figure 8. EFFECT OF MMH EXPOSURE ON HEMOGLOBIN IN MONKEYS.

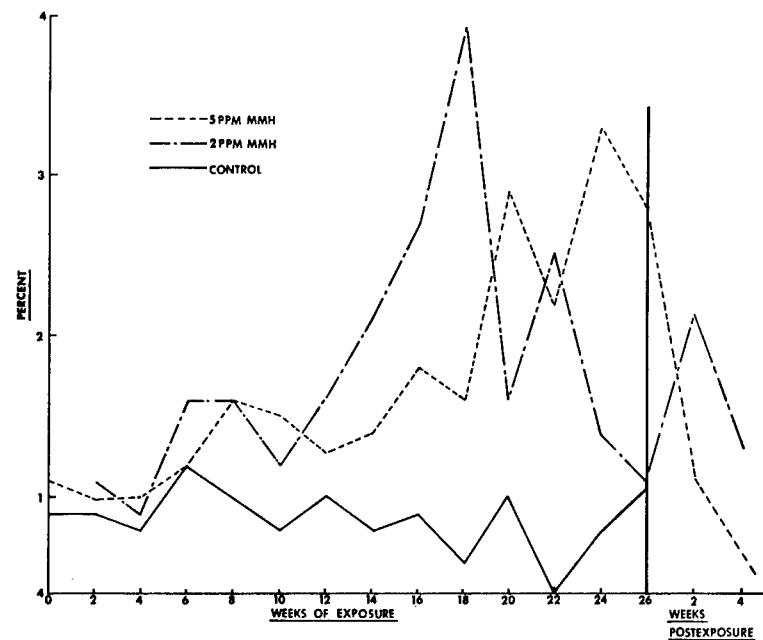


Figure 9. EFFECT OF MMH EXPOSURE ON RETICULOCYTES IN MONKEYS.

At 4 weeks, HCT, HGB and RBC values for the 5 ppm MMH exposed monkeys were significantly different, at the 0.05 level, when compared with control animal measurements. A rise in reticulocyte counts at 8 weeks and at all subsequent biweekly intervals during exposure was enough to produce statistical differences between test and control values. The overall net depression of these hematology measurements was 15% below preexposure values.

Probably the most obvious evidence of MMH effect in the 2 ppm exposed monkeys can be seen in the graph of the RBC values (figure 8) where a maximum depression of 15% occurred at the 16-week interval.

The rather noticeable rise in reticulocyte counts (figure 9) for monkeys exposed to the 2 ppm level between the tenth and eighteenth weeks was caused by the abnormally high reticulocyte values recorded for one monkey. This animal was surgically fitted with an implanted electrode to allow for EEG measurement during the course of the experiment. Subsequent infection, although not endangering life, was enough to adversely influence the mean reticulocyte values recorded for this group.

The quantitative differences in methemoglobin production between test and control dogs are shown in figure 10. The mean methemoglobin values recorded for dogs exposed to 5 ppm MMH are significantly elevated above those of the 2 ppm exposed and control animals throughout the course of the study. Similarly, but to a lesser extent, the methemoglobin values of the 2 ppm exposed dogs reflect positive differences from the control values. This graph portrays a reasonably clear dose response relationship.

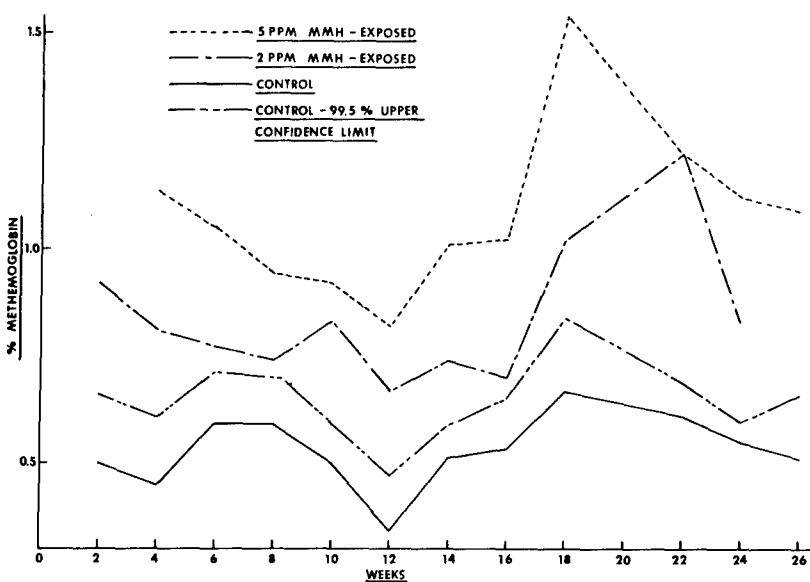


Figure 10. MEAN METHEMOGLOBIN VALUES, EXPOSED AND CONTROL DOGS.

Although the pattern of methemoglobin formation in test monkeys is not clear, the appearance of Heinz bodies in the blood of these animals provided evidence that MMH reaction with monkey hemoglobin did occur.

Blood samples were collected from experimental dogs on a regular biweekly schedule and examined microscopically for the presence of Heinz bodies. Although a consistent pattern was not seen, Heinz bodies were definitely observed in the blood samples of all test animals during the course of this study. Control blood, by contrast, was negative.

Clinical chemistry data which consists of 16 separate determinations collected during the course of the study were screened and tested for trends of biological and statistical importance.

Examination of statistical data comparing mean values of 2 and 5 ppm MMH exposed monkeys with their controls revealed significant differences in a few cases, at the 0.05 level, for BUN and uric acid. These differences occurred only at the second and twelfth week sampling periods.

Both serum bilirubin and alkaline phosphatase levels were significantly elevated in dogs exposed to either MMH concentration from the second week on. The elevation of bilirubin and alkaline phosphatase levels is consistent with the previously described pattern of erythrocyte destruction and the function of the liver in responding to this abnormal condition.

Increased susceptibility of dog red blood cells because of the hemolytic influence of MMH was measured also by means of an erythrocyte fragility test. Figure 11 shows fragilograms for the exposed and control dog groups. Values plotted for each graph are the mean values of five monthly determinations in each case. There was very little variation between animals in each group over the entire period. There is a definite shift toward increased initial hemolysis of the blood of the 5 and 2 ppm exposed dogs when compared with controls. The hemolytic response is more pronounced for the 5 ppm exposed animals, thus demonstrating a clear dose response effect. For example, percent hemolysis at a 0.54% salt solution is nearly zero for the control but 14% for the 2 ppm, and 20% for the 5 ppm dogs.

Previous acute toxicity tests (Jacobson, 1955; Haun, 1968) have shown that CNS damage is caused by exposure to MMH. To study manifestation of this effect, 3 monkeys, one from each experimental group, were fitted with brain implanted electrodes prior to the start of the study. EEG measurements made were monthly during the course of the study. The results were entirely negative in regard to measurable differences between test and control monkeys.

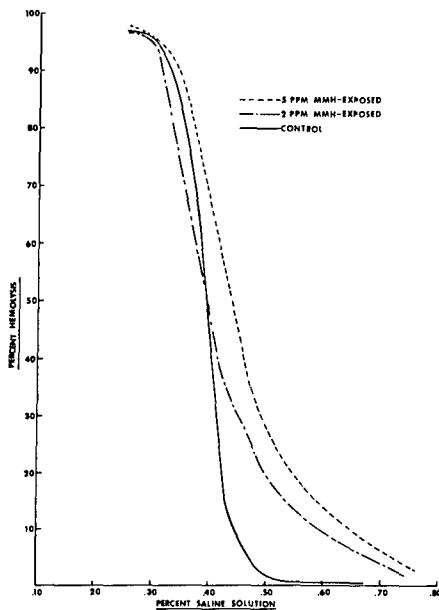


Figure 11. EFFECT OF MMH EXPOSURE ON RBC FRAGILITY IN DOGS.

### Pathology

To study possible pathologic changes produced in rats as a result of MMH exposure, one rat from each study group was sacrificed every month. Examination of six sets of histologic results revealed that splenic hemosiderosis was a common finding in rats from both exposure levels. An inconsistent finding was pigment deposition in kidney proximal tubules.

With the exception of the large animals previously mentioned, all exposed and control animals were sacrificed at 26 weeks and submitted for gross and histopathologic examination.

Gross pathology results which can be related to MMH exposure effect were black livers in all dogs at both exposure levels, and noticeably enlarged spleens in the majority of the mice exposed to 5 ppm MMH.

Bone marrow samples removed from test and control dogs showed very obvious differences in hemopoietic activity which was dose related. Visual examinations as well as comparison of colored photographs revealed that marrow from the 5 ppm exposed dogs was very dark red to black throughout, while the samples from the 2 ppm animals were mostly dark red. Control marrow was the normal yellowish white. Microscopic examination of these samples confirmed that there was a noticeable shift and increase in the erythroid and a decrease in the myeloid elements.

At sacrifice, heart, lung, liver, kidney, and spleen were harvested from all exposed and control rats and weighed to allow for calculation of organ to body weight ratios.

Statistically significant differences at the 0.01 level were noted when the kidney and spleen ratios for the 5 and 2 ppm exposed rodents were compared separately with control data.

Splenic engorgement resulting from increased production and destruction of red blood cells due to exposure accounts for real differences between exposed and control spleens. Until complete histopathology reports are available and analyzed, no final conclusions will be drawn concerning the biological significance of statistical differences between test and control kidney ratios. However, the suggestion is that enlarged kidneys may possibly be due to abnormal burden of red blood cells destroyed as a result of exposure to MMH.

Minimal histopathology information based on preliminary screening of tissues of only one animal of each species from each experiment revealed that dogs and mice showed a marked hemochromatosis of the liver with intrahepatic cholestasis. The blood pigments were in macrophages and Kupffer's cells aggregates both in portal triads and in the parenchyma. Some pigment was also found in liver cells. The cholestasis in the dogs was limited to the canaliculi but in the mouse was also seen in small ducts.

Dogs and mice also had increased pigmentation of the renal tubules at both exposure levels. Rats also showed this change at the high but not at the low dosage. No lesions were noted in the primates.

The kidneys of dogs and monkeys, exposed and control, are being processed for electron microscopic examination at this time. The results should clarify the question of kidney pathology, at least in large animals.

All in all, the results of this study justified additional exposures at lower concentrations to find the no-effect levels of MMH.

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## DISCUSSION

DR. SCHEEL: Were any glucose tolerance studies done on these animals during your exposure time?

MR. HAUN: No sir, we did no glucose tolerance tests; however, we did measure glucose as part of our routine battery of clinical tests and there were no changes.

DR. BACK: This was somewhat surprising since MMH does have a marked effect on blood glucose at least in acute exposures.



The volunteers were fully informed of the nature of the test, the toxicology of MMH, and the safety procedures established for the conduct of the exposure. They were given pretest physical and neurological examinations and were monitored for 60 days post-exposure. The physical examinations were either Class II USAF or Class II FAA flying physicals. These subjects were reasonably representative of the type of people who might be expected to work in fuel manufacturing and handling, ranging in age from 23 to 44 years old (average age 31) and included nonsmokers, reformed smoker, and heavy smokers. The volunteers were all male employees of SysteMed Corporation and included Caucasian and Negro, technical and professional personnel.

In order to assure the safety of the volunteer exposure subjects, one subject was exposed first to 50 ppm MMH for 10 minutes (a little more than 1/2 of the proposed limit value) and observed for 2 weeks postexposure before a second subject was exposed to 70 ppm MMH for 10 minutes. When the second subject showed no effects and was still negative after 2 weeks, the remaining subjects were exposed to the 90 ppm MMH concentration. Five volunteers were exposed to the 90 ppm MMH level and 7 subjects in all were tested in this study.

The exposures were conducted by inserting the subject's head into a Rochester Chamber through a rubber diaphragm located in one plastic panel as shown in figure 1. The subjects, as shown in figure 2, wore a standard aircraft radio headset for communicating with the attending personnel. The MMH concentrations, which were continuously monitored, were established and stabilized in the chamber and then the subject inserted his head for 10 minutes and his sensations were recorded. For comparative purposes, 2 concentrations of ammonia vapors (50 and 30 ppm) were established in the chamber and the same volunteers made similar observations of odor and irritability.



Figure 1. VOLUNTEER WITH HEAD IN EXPOSURE CHAMBER,  
EXTERNAL VIEW



Figure 2. VOLUNTEER WITH HEAD IN EXPOSURE CHAMBER,  
INSIDE VIEW

As mentioned earlier, we were concerned with the possibility of a 90 ppm MMH concentration causing excessive lacrimation or bronchospasm. None of the subjects experienced any difficulty of this nature. Their eyes were observed through the chamber window and examined carefully after removing their head from the exposure port. Most of the subjects had increased moisture in the eyes but not overt enough to cause overflow tearing. There was also slight erythema in the eyes of some subjects. Table I shows the individual responses to the irritant quality of MMH as compared to 30 and 50 ppm ammonia. They did not know the ammonia concentrations, and the tests with the 2 ammonia concentrations were given randomly. The 90 ppm MMH concentration was slightly more irritant than 30 ppm  $\text{NH}_3$  but considerably less than the 50 ppm  $\text{NH}_3$  atmosphere. Most subjects rating 90 ppm MMH as scale factor 1 referred to a slight tickling sensation of the nose while one subject had a slight nasal drip. The subjects were variable in respect to describing the mild irritation as affecting the eyes, nose or in one case an unpleasant taste sensation. Odor comparisons for the 3 test atmospheres are shown in table II. The 90 ppm MMH odor was found to be less noticeable or offensive to most subjects than either  $\text{NH}_3$  concentration. One of the 7 test subjects was not included in this compilation because he had a poor olfactory response and was unable to smell any of the experimental atmospheres.

TABLE I  
SUBJECTIVE COMPARISONS OF IRRITATION FROM  
MMH AND AMMONIA

<u>Subject No.</u>	<u>30 ppm NH<sub>3</sub></u>	<u>50 ppm NH<sub>3</sub></u>	<u>90 ppm MMH</u>
1	1	2	2
2	0	2	2
3	0	0	1
4	0	1	1
5	-	2	1
6	1	2	1

## IRRITANT SCALE (NASAL AND EYE)

<u>Degree</u>	<u>Intensity</u>	<u>Description</u>
0	No Irritation	Not detectable
1	Faint	Just perceptible, not painful
2	Moderate	Moderate irritation
3	Strong	Discomforting, painful, but may be endured
4	Intolerable	Exceedingly painful, cannot be endured

TABLE II  
SUBJECTIVE ODOR COMPARISONS OF MMH AND AMMONIA

<u>Subject No.</u>	<u>30 ppm NH<sub>3</sub></u>	<u>50 ppm NH<sub>3</sub></u>	<u>90 ppm MMH</u>
1	3	4	4
2	4	4	3
3	4	4	3
4	4	4	4
5	-	4	3
6	3	4	3

## SCALE OF ODOR INTENSITY

<u>Degree</u>	<u>Intensity</u>	<u>Description</u>
0	No Odor	No detectable odor
1	Very Faint	Minimum, but positively perceptible odor
2	Faint	Weak odor, readily perceptible
3	Easily Noticeable	Moderate intensity
4	Strong	Highly penetrating
5	Very Strong	Intense effect

The mean results of pre- and postexposure clinical chemistry determinations were evaluated and found unchanged as shown in table III. All individual values were within the normal range for this laboratory. There were no changes in the individual or group values resulting from the exposure to MMH. Since MMH at higher concentrations has been shown to produce in some species a transient increase in methemoglobin with a resultant Heinz body anemia, hematology studies were also conducted. The group mean pre- and postexposure values are shown in table IV. Although not shown in this table, methemoglobin values were determined preexposure and twice postexposure. The postexposure measurements were made immediately and 5 hours later. There was no increase in the endogenous methemoglobin level. However, Heinz bodies did appear in the blood of the exposed subjects ranging from 3 to 5% by the 7th postexposure day and disappearing during the next week. The appearance of the Heinz bodies was not accompanied by any signs of anemia or reticulocytosis.

TABLE III  
MEAN CLINICAL CHEMISTRY MEASUREMENTS  
ON MMH EXPOSED HUMAN SUBJECTS

Test	Units	Preexposure	1 Day	7 Days	14 Days	60 Days
Sodium	meq/L	145	145	145	144	140
Potassium	meq/L	4.5	4.6	4.5	4.4	4.2
Cholesterol	mg %	209	210	210	202	---
Calcium	mg %	10.4	10.4	10.3	10.3	10.3
Inorg. Phosphorus	mg %	3.4	3.2	3.5	3.3	3.8
Tot. Bilirubin	mg %	0.6	0.7	0.6	0.6	0.6
Total Protein	gm %	7.7	7.6	7.6	7.6	7.5
Albumin	gm %	4.9	4.9	4.9	4.8	4.4
Uric Acid	mg %	7.2	7.1	7.0	7.0	6.6
BUN	mg %	15	14	14	15	13
Glucose	mg %	100	104	95	96	104
Creatinine	mg %	1.1	1.1	1.0	1.2	1.1
Chloride	meq/L	111	108	108	107	106
LDH	units*	107	108	112	94	94
Alk. P'tase	K. A. U. **	12	12	12	10	10
SGOT	K. U. ***	23	24	25	21	25

\* Technicon® Units

\*\* King Armstrong Units

\*\*\* Karmen Units

TABLE IV  
HEMATOLOGY MEASUREMENTS ON MMH EXPOSED HUMAN SUBJECTS  
(Group Means)

<u>Test</u>	<u>Units</u>	<u>Preexposure</u>	<u>1 Day</u>	<u>7 Days</u>	<u>14 Days</u>	<u>60 Days</u>
HCT	vol %	46	46	44	45	44
HGB	gm %	15.0	14.8	14.7	14.7	14.2
RBC	millions	5.3	5.3	5.3	5.2	5.1
WBC	thousands	9.3	8.4	8.9	9.7	9.0
Reticulocytes	% RBC	0.8	0.8	0.7	0.9	0.9
Heinz Bodies	% RBC	0	0	4	1	0

TABLE V  
DETERMINATIONS OF VENTILATORY CAPACITY  
ON MMH EXPOSED HUMAN SUBJECTS

<u>Test</u>	<u>Units</u>	<u>Preexposure</u>	<u>Immediate Postexposure</u>	<u>60 Days</u>
FVC	ml	4771	4743	4644
FEV <sub>1.0</sub> "	ml	3808	3717	3694
FEV <sub>1.0</sub> "/VC	%	80	79	80
MBC	L/min	155	170	168
MMF	L/sec	3.95	3.74	3.72

Spirometric measurements of pulmonary mechanical functions were also made with no significant changes as shown in table V. The improvement in maximum breathing capacity probably represents a learning process. One subject had a decrease in all measured parameters beginning 24 hours postexposure. He also displayed symptoms of respiratory infection and was given a chest x-ray which showed acute bronchitis in the lower left lung field. A followup chest plate taken one week later was negative and his lung volume began returning to baseline values. Another subject whose baseline lung volumes were low stopped smoking and showed a gradual increase in these tests during the postexposure observation period.

The results of this study demonstrate that the tentative 90 ppm MMH 10 minute emergency exposure limit has an adequate safety factor and did not produce bronchospasm or severe lacrimation. It is recommended that the tentative status of this EEL be removed.

## DISCUSSION

MR. WANDS: Not a question but just a comment. Doug, I want to thank you very much for continuing your studies on this. We did appreciate your original data being made available to the Committee so they could get some reasonable EEL's for this compound which is a critical ingredient in many of our rocket programs in this country and does present some significant short-term occupational hazards and now this last follow-up will be most useful to the Committee, and I'll look forward to having, if I may, a copy of the data to present to them at their next meeting.

DR. MAC EWEN: I shall be pleased to provide you with a copy of the data.

DR. BACK: I'm a little bit confused about the data, Doug. I don't like to see all of this kind of data lumped, for the simple reason that the parameter, one parameter you were looking at was most significant was Heinz body formation. Now out of all those subjects, how many subjects had Heinz body formation and how long did it last in those that did? You can't tell from the slide.

DR. MAC EWEN: No, but if I made a slide for each of the individuals and for each of the things measured, we'd be here until tomorrow afternoon, I suspect. I presented the data as group means to conform to the program time limits. The highest Heinz body measurement for an individual was 5%. The range was 3 to 5% of the RBC's and they appeared a few days after the exposure. They were lower at two weeks and there were no Heinz bodies at the 60-day postexposure point. I don't want to get into the discussion of Heinz body anemias but I would like to make a couple of comments. In our work here with monomethylhydrazine and NF<sub>3</sub>, which also produces Heinz body anemia, it appears to us that the Heinz body is some form of denatured heme protein in those cells and that the cells are on their way to destruction. We find the Heinz bodies extracellular in the blood as well as intracellular.

DR. SCHEEL: What I would like to ask, even though you didn't mention it, was there any change in any subject on kidney function, especially with regard to sugar or albumin in the urine?

DR. MAC EWEN: I didn't mention it, but we did examine the urines of these people. No, there were no changes as indicated by glucose, albumin or by microscopic examination. We also ran BSP's on several of the subjects, which I'm not overly fond of but the Review Committee asked us to do them, and they were also normal.

## MONOMETHYLHYDRAZINE EFFECT ON BLOOD, IN VITRO

Harold F. Leahy

SysteMed Corporation  
Wright-Patterson Air Force Base, Ohio

### INTRODUCTION

Monomethylhydrazine (MMH), an important reactant in many rocket propellant systems, is known to be highly toxic (Jacobson et al, 1955). Although the acute toxicity is related to central nervous system involvement (Haun et al, 1969) a concurrent effect of some physiological importance following exposure is the oxidation of hemoglobin to methemoglobin (Fortney and Clark, 1967).

Similar reactions of a coupled oxidative nature have been observed with other reducing compounds such as ascorbic acid (Lemberg et al, 1939) and phenylhydrazine (Jandl et al, 1960). Species differences of methemoglobin levels following MMH exposure, both *in vivo* and *in vitro*, as observed by Clark and De La Garza (1967), have a parallel in the observed effect of acetanalid, acetophenetidin (Lester, 1943) and diamino-diphenyl-sulfone (Francis, 1949).

The involvement of oxygen in cooxidation reactions has been reported previously for phenylhydrazine (Beaven and White, 1954) ascorbic acid (Lemberg et al, 1941b) and other reducing compounds, but only alluded to in the case of MMH.

Benzene and nitrogen have been identified among the products found during the cooxidation of phenylhydrazine and hemoglobin (Beaven and White, 1954). In the study of Dost et al (1964) on the metabolic fate of UDMH and MMH, a labeled volatile compound was tentatively identified as methane  $^{14}\text{C}$ . In the air oxidation of MMH the major breakdown products observed have been nitrogen and methane (Vernot et al, 1967).

The present study was conducted, first to confirm previous work with respect to the identification of the hemoglobin compound formed and species difference in methemoglobin level; second, to determine the involvement of oxygen in the reaction; and third, to identify the major breakdown products of MMH in the *in vitro* system.

## MATERIALS AND METHODS

MMH was purchased from Matheson and, once opened, stored under nitrogen. Fresh solutions of 0.1 M MMH in 0.2 N HCl were prepared daily. Heparinized blood was obtained from stock animals and, except for the human study, was pooled from five or more animals.

Unlimited aerobic reaction mixtures were incubated at room temperature with 5 ml of blood in a 50 ml beaker, having a humidified air supply, with continuous stirring. Pooled canine blood was calculated to contain approximately 44 micromoles heme per 5 ml, and typical reaction mixtures were made by addition of sufficient MMH solution to give the required molar ratio of heme/MMH, (for example 2:1, heme/MMH required 0.22 ml of 0.1 M MMH per 5 ml canine blood).

Limited aerobic and anaerobic reactions were performed in sealed 50 ml serum vials. The blood in the latter case was physically deoxygenated by alternate evacuating and replacing of headspace gas by helium at least six times. Oxygen was added by syringe when desired. A few experiments were performed using washed erythrocytes and hemoglobin solutions at approximately physiological hemoglobin levels.

Reaction rates were observed by sequential spectral analysis using a Perkin-Elmer 350 spectrophotometer operated in absorbance mode in the visible region. At each time period a 0.1 ml aliquot was diluted 1/100 in M/60 phosphate buffer (pH 6.6) and the spectrum was recorded both before and after the addition of a drop of 10% KCN. The buffer was deoxygenated for spectra of the anaerobic reactive mixtures and kept in serum stoppered tubes. Methemoglobin was quantitated essentially by the method of Evelyn and Malloy (1938) but modified to allow a correction for turbidity loss on addition of KCN. The absorbance at 720 nm is subtracted from that of 630 nm before conversion of the latter to methemoglobin.

Analysis of the overhead gas was performed on a 5A° molecular sieve column operated at 62 C using a Varian A90 P G.C. with a thermal conductivity detector, and also on a Poropak Q column operated at 120 and 160 C using an F & M 700 gas chromatograph with dual flame ionization detectors.

## RESULTS

As the aerobic MMH-hemoglobin reaction proceeded the mixture turned from bright red to purple brown in appearance, during which an observable release of gas occurred. With high MMH to heme ratios, more than 2:1 on a molar basis, rapid denaturation and precipitation followed. At lower ratios the rate of hemoglobin conversion was directly proportional to the MMH level.

Identification of the compound formed as methemoglobin was based on spectral analysis of the compound itself and a number of the reaction products of the compound. The spectrum of normal hemoglobin (oxygenated and reduced) and methemoglobin (ferri-cyanide) (figure 1) can be compared with the spectrum of an aliquot taken from a reaction mixture (figure 2) using dog blood. The latter spectrum resembled that of a mixture of methemoglobin and cyanmethemoglobin.

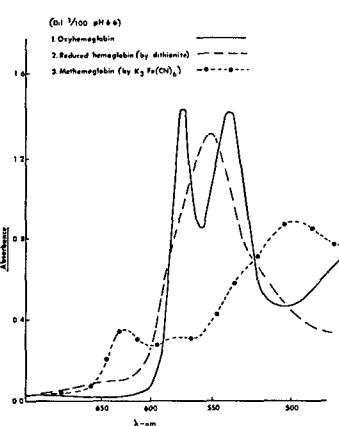


Figure 1. SPECTRAL CHARACTERISTICS OF HEMOGLOBINS.

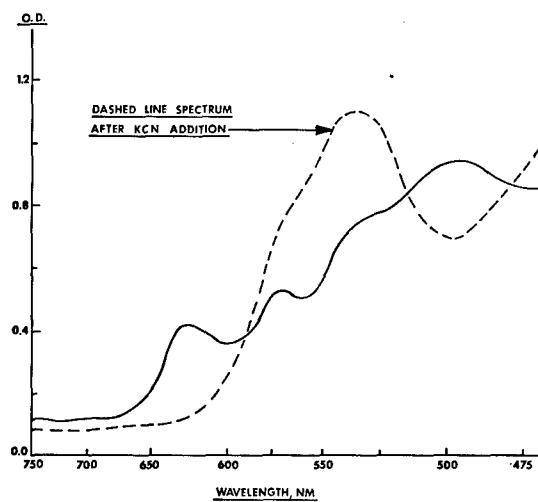


Figure 2. CONVERSION OF HEMOGLOBIN TO METHEMOGLOBIN BY MMH.

A pH shift to alkaline caused some loss of the 630 nm peak (in one experiment approximately 1/3 decrease with a shift from pH 6.4 to 7.9).

Addition of dithionite reduced the compound to hemoglobin (figure 3). This reduced hemoglobin in turn was capable of reacting with both CO to form carboxyhemoglobin and strong alkali to form protohemachrome. These reactions are characteristic of methemoglobin (ferricyanide) and support identification of the MMH-hemoglobin reaction product as methemoglobin but distinguish it from choleglobin, the ascorbic acid-hemoglobin reaction product of similar spectral nature.

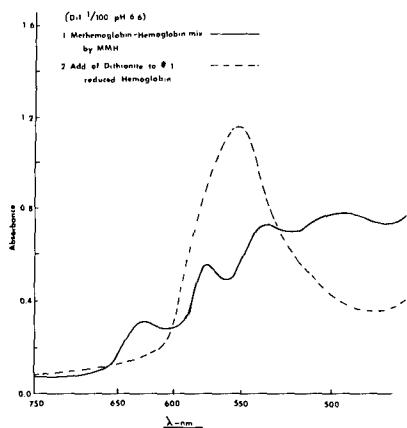


Figure 3. EFFECT OF DITHIONITE ON MMH-HEMOGLOBIN REACTION PRODUCT

The spectrum obtained from anaerobic reaction mixtures (figure 4) is that of reduced hemoglobin with only a slight amount of methemoglobin present. Following the reaction in time (figure 5), the mixture appears stable for at least two hours while kept anaerobic, but highly reactive on exposure to air.

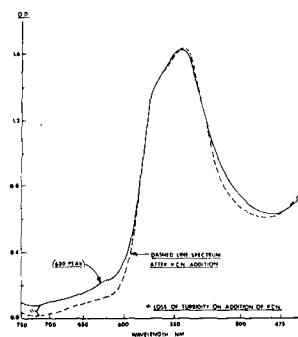


Figure 4. EFFECT OF MMH ON REDUCED HEMOGLOBIN TO METHEMOGLOBIN.

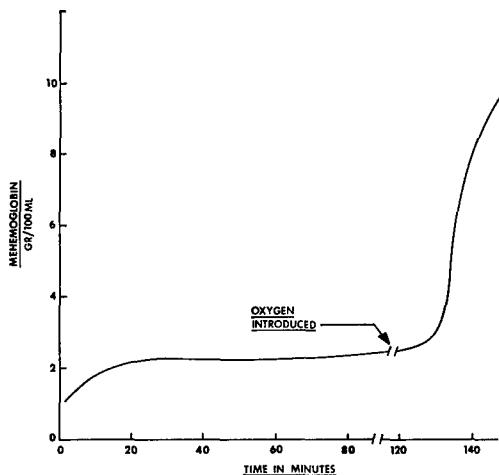


Figure 5. METHEMOGLOBIN FORMATION RATE.

In unlimited aerobic reactions of MMH with dog blood (figure 6) both the rate of methemoglobin formation and the total reached are directly proportional to the original concentration of MMH, although limited to conversion of about 75 to 80% of the original hemoglobin. The molar ratio of reactants approaches 2:1, heme/MMH at low MMH concentration.

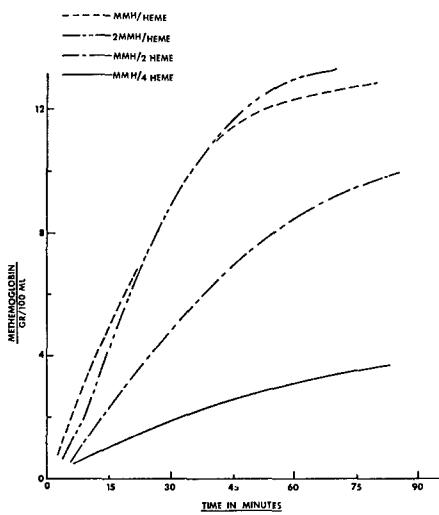


Figure 6. EFFECT OF VARIATION IN MMH CONCENTRATION ON METHEMOGLOBIN FORMATION RATE.

The blood of other species appears to have different equilibrium levels, beyond which denaturation proceeds, or methemoglobin reduction keeps pace with formation. A histogram drawn from data of a series of parallel experiments (figure 7) demonstrates the species differences.

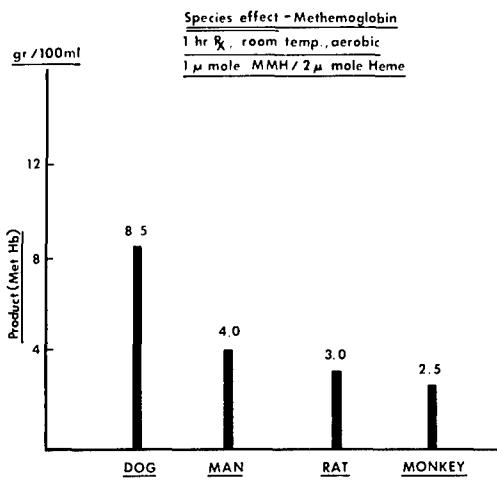


Figure 7. EFFECT OF MMH ON IN VITRO METHEMOGLOBIN FORMATION FOR VARIOUS SPECIES.

No major differences were observed with respect to reactivity of whole blood, washed and resuspended erythrocytes, or clarified hemoglobin solutions of approximate physiological hemoglobin concentrations.

A gas chromatographic analysis of the headspace gas in sealed reaction vessels showed nitrogen and methane as products while oxygen was consumed. Although the identification is by comparative retention time only, methane was observed on two different columns. Approximately 80% of the nitrogen and 20% of the carbon of the MMH was accounted for. No other volatile compounds were observed even with direct injection of the reactive mixture.

## DISCUSSION

The results of these experiments support previously reported work on hemoglobin-MMH reaction with respect to both product (methemoglobin) formation and species differences. Measurable methemoglobin occurred over a relatively narrow range of MMH concentration. Denaturation of the globulin and precipitate formation occurred when MMH was in excess possibly due to reaction with sulfhydryl groups of the protein.

Ascorbic acid reactions show similar effects, and denaturation also appears to occur rapidly after 75% conversion of hemoglobin to choleglobin (Lemberg et al, 1941a).

The necessity for the presence of oxygen for the cooxidation reaction was shown for MMH. This was similar to ascorbic acid, phenacetin, and the arylhydrazine reactions. The effect of various  $O_2$  concentrations on rate of conversion was not determined due to difficulties in quantitation of oxygen remaining as the reaction proceeded. In other similar reactions the rates were increased where there was a low  $O_2$  pressure in spite of the fact that the reaction requires oxygen. This would seem to support the idea that an intermediate compound between reduced and fully oxygenated hemoglobin is the substance actually converted to methemoglobin.

Lemberg et al (1941b) suggest that two hydrogen atoms are transferred from ascorbic acid to oxyhemoglobin, giving rise to an unstable hemoglobin-hydrogen peroxide complex which in turn breaks down. Keilin and Hartree (1945) have demonstrated that catalase, although present in great excess, will not protect hemoglobin from oxidation in the case where a primary oxidation results in hydrogen peroxide formation.

Jandl (1960) along with Bevan and White (1954) have pointed out that most hemolytic drugs are resonating aromatic compounds similar to reversible redox systems. Substances of this nature could speed electron transfer from ferrous heme groups and from sulfhydryl groups to molecular oxygen by providing partially oxidized intermediates, including free radicals, stabilized by resonance.

The reactants hemoglobin and MMH are both capable of auto-oxidation to the same compounds formed in the coupled reaction. In this reaction, as with the other cooxidation reactions, each reactant appears as the catalyst for the destruction of the other. Either proposed theory alone or a combination could explain the overall reaction. The results of the present study are not in conflict with either proposed mechanism.

#### SUMMARY

The reaction of MMH with hemoglobin of various animal species at near equimolar amounts has been investigated. Previous work with respect to compound formed and species differences of level of hemoglobin oxidation was confirmed. The necessity for the presence of oxygen during the reaction was demonstrated, and during the course of the reaction nitrogen and methane were liberated.

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## DISCUSSION

MR. WANDS: I'd like to ask if Mr. Leahy considers that the release of methane might be a mechanism for monitoring the actual amount of MMH that may have been absorbed by an exposed person?

MR. LEAHY (SysteMed Corporation): I don't know if I would be qualified to answer that question. We didn't attempt it in this rather low level of methane but it is a relative easy compound to see on the G.C.

DR. BACK: Frank Dost can probably say something about that since they did study the metabolism of the compound and they did find methane release. If you'll comment on that, Frank?

DR. DOST: Yes, the interesting thing about the release of methane and CO<sub>2</sub> from animals which had been administered labeled MMH was that as the dose was decreased, the percentage of the administered dose converted to the volatile product rose until we got down to trace levels and we were finding on the order of 90% conversion of the carbon to methane and CO<sub>2</sub> and the ratios between the two products at any given time in their period of metabolism after a single dose remains the same regardless of the dose and our contention is and this is supported, to some extent, by work which has subsequently been done by Don Reed at our place and by people at NIH with other hydrazines is that this is actually a function of the intoxication reaction rather than the detoxication reaction.

MR. LEAHY: Methane is a bacterial by-product in the intestines and it might be a problem for the analyst.

DR. DOST: There is no question that a great deal of methane is produced by intestinal microorganisms. I'm speaking strictly of the labeled methane. We found ourselves with one foot in that very trap at one time. The question that I have is that if I recall the two spectra of what I might call standard methemoglobin and the methemoglobin produced by MMH, these spectra differ to some extent, did they not?

MR. LEAHY: The difference is the presence of some oxyhemoglobin still in the mixture. You can get the same type spectrum by serial dilution of a ferricyanide produced methemoglobin with oxyhemoglobin to a point where you run out of oxidant. I had one spectrum where you could superimpose the two curves and you couldn't see the difference. We were limited to only about a 75% conversion to methemoglobin, the rest was oxyhemoglobin.

MR. TOLIVER: That was a very good piece of work in kinetics, at least that's what it appears to me. But did you attempt to find out whether or not it is a first order kinetic reaction? Did you observe this to be the case, that one got products as a function of the presence of one species? If you can answer this kind of question then the other kinds of questions that are coming up can be answered.

MR. LEAHY: You mean a certain percentage of oxyhemoglobin and reduced hemoglobin?

MR. TOLIVER: Yes, where the rate was a function of concentration of oxygen or one species present. This would make it a first order reaction.

MR. LEAHY: We're just getting to that point of trying to quantitate the oxygen and we were getting reproducible peaks but the question was how much oxygen is still now attached in a form of oxyhemoglobin, what effect does methemoglobin have on the release of the oxygen, and I got so bound up in trying to find out how much oxygen was present, I didn't have time to go into that area yet.

MR. TOLIVER: Let me say again, it was a very good piece of work but you do plan to follow on to find out the kinetics of the reaction, I trust?

MR. LEAHY: We hope to continue, it's a part-time operation.

DR. THOMAS: Could we have the very last slide back on the screen? Remember last year I asked the question, since we know that monomethylhydrazine tears up the dog kidney fairly well, we are wondering about the human. Is he more like the dog, the monkey, or the rat? You ran through that slide a little bit too fast, and I think the message is right there.

MR. LEAHY: This slide shows the comparative peak in 60-minute reaction time of methemoglobin produced. Now, both the rat and the monkey had reached that level at an earlier time. They had peaked at 30 minutes, but the dog is still progressing and 15 or 20 minutes later it reached 12 grams percent. The rat did not nor the monkey did not, and the human went a little further, about half-way, it followed on through. The rat and monkey had about gone as high as they were going. MMH was still present in the rat and monkey blood because I tried mixing it with dog blood and I had oxidation immediately with dog blood. On allowing the rat blood MMH mixture to stand overnight, I ended up with the same amount of methane from the rat as I had gotten from the dog, but there was no further methemoglobin. In fact, the methemoglobin had all been reduced in the rat blood by the next morning. I couldn't find any more. The dog blood usually goes on and is destroyed at that level. If that answers the question.

MR. WANDS: I wonder if perhaps what Dr. Thomas was trying to get after was the basic question of whether or not man is a dog in terms of response to MMH or whether he is a rat or a monkey. Which animal most nearly resembles this? Now, I would like to come back to that slide again, not necessarily show it, but the concepts that were there, and ask Mr. Leahy if he feels that the data as presented there, the picture that is presented in that slide, is a true representation of all of the time responses that you mentioned and then I would also like to go over Charlie Haun's paper and ask if perhaps in his work he doesn't see that the dog is much more sensitive to this than any of the other species that he looked at. So, on the basis of the two papers here we are seeing things where the dog is a much more sensitive species than either the man or the other two animals. Is this a correct impression?

MR. LEAHY: Yes, the dog is the most sensitive of the animals that were studied, although the human is a close second to the dog given a little more time in the reaction rate. The human had not reached the equilibrium at this point, whereas the rat and the monkey had approached an equilibrium, they did not go higher in their methemoglobin formed. But the humans only approached about 50 to 60% the level of methemoglobin of the dog with the same amount of monomethylhydrazine.

MR. WANDS: The maximum values would be somewhat different from those shown in this graph?

MR. LEAHY: The dog would go up to about 12% and the human would go up to about 7-8% for that level.

DR. THOMAS: I'm really forcing my hand here a little bit. I think the inference which I'm trying to make here is that when we look at brief exposures, I think man is going to be a little bit more protected than the dog, but I don't think it will have any effect on chronic exposures, and given enough time to equilibrate with the same atmosphere, I think man is going to have hematological problems if we pick the wrong TLV's, and I had a little discussion with Dr. Jacobson on this. I told him we are now running MMH at 0.2 ppm both for five days a week, 6 hours a day and continuously, and I wondered if he would care to comment on some of the concepts which the Committee is using in setting TLV's and the amount of safety factors which are thrown in because of the nature of the compound? I almost feel uncomfortable with a 0.2 ppm TLV at the present when you know that this is a compound which has marked cumulative effects, and the target organ in chronic exposures is, I believe, the hemopoietic system.

DR. JACOBSON: I can't comment from recent experience in the recommendation of Threshold Limit Values, but going back a few years, I think Henry Smyth has given the most effective critique of this and he has pointed out that the safety factor varies greatly from one compound to the next and that in a few cases, the safety factor seems to be less than one. Since the Threshold Limit Value is a little higher than some effect levels in animals, but to the extent there is any system, any rationale, to this variation in safety factors, I believe it is essentially this. What is the risk in extrapolating from animals to man? That risk depends on at least two factors. One is the soundness of the

work in animals and the soundness of the extrapolation to man. Another is what are the effects that the Threshold Limit Value should protect against? If the Threshold Limit Value protects against a mild transient effect then one can take great risks in extrapolating to man, a large safety factor is not needed. If, however, the effect that the Threshold Limit Value should protect man against is a permanent and irreversible, possibly terminal, effect for example, malignant tumors, then very large safety factors are needed and in our brief discussion of this general problem out in the hall, my comment was in that comparing monomethylhydrazine with dimethylhydrazine, I would want a much larger safety factor with monomethyl than I would with dimethyl. Does that answer your question?

DR. THOMAS: We all appreciate, in this room, how much difference you can get by taking the same basic compound and just juggling methyl groups around. Some of you might not be familiar with earlier work where people were studying symmetrical dimethylhydrazine and drew some hasty conclusions about the toxicity of it. They said it's relatively nontoxic. Several grams per kilo doses were administered, but the animals weren't observed for 7 days. Now symmetrical dimethylhydrazine is different from unsymmetrical dimethylhydrazine in that the animals die only a week later, but they do die.

DR. JACOBSON: If I could extend that a little bit further. The first hydrazine derivative much was known about in the way of toxicity was phenylhydrazine. And toxicologically phenylhydrazine is an aniline derivative, it's amino aniline. Then along came hydrazine and the assumptions were made that hydrazine would act toxicologically much like phenylhydrazine and this was a completely misleading assumption. Hydrazine has a much different type of action. It causes CNS stimulation to the point of convulsions and, of course, it causes adverse effects on the kidney, liver, and can cause contact dermatitis. Then the methylhydrazines came along, the first one was a dimethylhydrazine, the unsymmetrical dimethylhydrazine, and it was assumed that dimethylhydrazine would act very much like hydrazine, and very soon in the game it was discovered it was a convulsigen like hydrazine, but did not seem to cause the hepatotoxic effects, or for that matter the kidney effects that hydrazine caused. So that the addition of two methyl groups seems to make the compound a more potent convulsigen, but less potent in other respects. Then the withdrawal of one of those methyl groups, many predicted, would give a compound that was somewhere in between hydrazine and dimethylhydrazine, and in fact I believe, that methylhydrazine is a still more potent CNS stimulant and brings us back to phenylhydrazine in its effect on the blood. So in one respect at least, methylhydrazine and phenylhydrazine are alike, but only in that one respect.

MRS. PINKERTON (Aerospace Medical Research Laboratory): The monkeys that you used, or the monkey blood that you used, Mr. Leahy, was what? Was it macaque, Rhesus monkey blood, or was it squirrel monkey?

MR. LEAHY: Rhesus monkey.

MRS. PINKERTON: Did you do any work with squirrel monkey blood?

MR. LEAHY: No, that was limited work. We had five or six monkeys waiting to go in the dome.

MRS. PINKERTON: Well, in view of the toxicity data from the squirrel monkey which came up, wouldn't it be sort of interesting to compare the two monkeys, in this respect to see if they're different?

MR. LEAHY: We'll have to put that down on our long list of things to try.

DR. MAC EWEN: Ken, may I comment on that?

DR. BACK: Yes.

DR. MAC EWEN: Yes, we did find the squirrel monkey to be the most sensitive animal to the acute toxicity but I'm not sure it was because it was truly the most sensitive or because it is so highly parasitized that it is sensitive to anything, even handling. By the time you sample it a few times it seems to sort of deteriorate whether you expose it to the hydrazine or not. Our pathologists are always complaining every time they look at a squirrel monkey, or sometimes they're like stamp collectors, they just love to look at it because they find so many parasites and they describe them in every organ and every orifice.

MR. HAUN: Could I make a comment, Ken? Earlier in the game when we were doing acute work we found squirrel monkeys unsuitable because of these parasitic infections and during a paper the other day, I think one of the veterinarian pathologists mentioned that his had infestations of microfilaria. This was the case in many of the squirrel monkeys that we used in acute work, so we decided not to use the squirrel monkey after that. They are very poor test subjects.

MR. VERNOT: If I may make a few comments on the genesis of the work that Mr. Leahy has done on blood. We, of course, wanted to elucidate some of the toxic effects of MMH on blood which we had already seen and check the work that had been done down at SAM, but we also wanted to check out a couple of other items. It has been reported that there have been complexes formed between MMH and blood and we wanted to see whether we would be able to see those spectrophotometrically at reasonable hemoglobin to MMH ratios and we were not able to do it. The most interesting part of the thing chemically was the fact that you get methemoglobin, supposedly an oxidation product of hemoglobin, from oxidizing compounds like  $\text{NF}_3$  and other oxidizing materials, ferricyanide, but the hydrazines are not oxidizing compounds, they are rather strong reducing compounds, and we thought we might be able to find out what the mechanism was of the formation of an oxidized hemoglobin from a strong reducing agent. I don't think we were able to do this. It has been postulated that hydrogen peroxide is the active agent and it is known that hydrazines do oxidize by peroxide routes, but we've found, and I guess everybody else who has ever tried it has found that you can dump fantastic amounts of hydrogen peroxide in the blood and not form one jot of methemoglobin; except Mr. Leahy has told me that if you form your peroxide in situ then you can do it, and I'm not sure what the significance of this is, it doesn't make sense to

me chemically. But these are the problems, some of the things we thought we were going to resolve or wanted to resolve and we were never able to resolve.

MR. LEAHY: With reduced hemoglobin, that is deoxygenated, and with very slow addition of peroxide as Kylin and Hartree recommended and going back and reducing it some more, we produced some methemoglobin, but it could be argued that we were working with an in-between solution and that it oxygenated by itself at the low level; however, a very slight amount of methemoglobin was produced on the addition of peroxide.

MR. VERNOT: A significant amount?

MR. LEAHY: Not tremendous, but more so than when I dumped it in when I saw absolutely nothing. We oxygenated and the mixture foamed for 5, 10 minutes as the catalase was eliminating oxygen because in the form of oxyhemoglobin it appears to be protected from methemoglobin formation, whereas even though oxygen appears necessary for the oxidation, if all the hemes are in the oxygenated form apparently you cannot form methemoglobin. You have to have some reduced heme also present in order to form methemoglobin.

MAJOR MAC KENZIE: I'd like to refer back to Mr. Haun's paper in discussing the amount of hematocrit as compared to the number of reticulocytes in the dogs that were exposed to 5 parts per million MMH. If you remember, at 5 parts per million there was a steady but real decrease in hematocrit towards the end of the experiment, in the last month. At the same time there was an increase in reticulocytes, there was also an increase in the number of nucleated RBC's and a decrease in the age of these nucleated RBC's, and I think this indicates even after six months of exposure that the dogs were becoming increasingly incapable of replacing the RBC's that they were losing, and they were falling behind in the game; and possibly in another two months they might have been in very serious trouble.

DR. BACK: I would like to refer to that paper too, because there was one thing mentioned that we haven't figured out and that's the relaxation of the nictitating membrane which makes you wonder about mechanism. If you remember all of the early work at the School of Aerospace Medicine, this compound was supposed to be, at least in a test tube, a monoamine oxidase inhibitor and if that were so, instead of getting relaxation of the nictitating membrane, you ought to get stimulation of the thing, because epinephrine and norepinephrine would be there. You know the nictitating membrane is sympathetically innervated in the dog, in any other animal that has one, and so relaxation means blocking of the sympathetic somewhere along the line and pharmacologically, monomethylhydrazine doesn't do this. But we did see it rather routinely in the 5 parts per million animals, and why I haven't the vaguest notion, but I think it's of interest, at least.

MR. HAUN: May I comment on that, Dr. Back? This is particularly interesting to me because during the course of our acute experimentation, we didn't see that in dogs at all. We watched them very carefully and recorded all the symptoms that were evident, and it just simply didn't happen in the acute exposures. Whether in the LC<sub>50</sub> survivors or LC<sub>90</sub> or LC<sub>10</sub>, we just simply didn't see that kind of thing at all.

DR. SLONIM: Mr. Leahy, you seem to indicate in your last reply that reduced hemoglobin tends to stimulate, or some reduced hemoglobin tends to stimulate the formation of methemoglobin. What would happen, would you get methemoglobin formed anaerobically or has anyone ever tried it in nitrogen atmosphere? Try to get to the thermodynamic reaction?

MR. LEAHY: Our one slide did show, in reduced hemoglobin that no apparent reaction was occurring during the period in which we kept it anaerobic, and on exposure to oxygen methemoglobin formed, and that's the point I was starting to attempt quantitating oxygen and that's where I got lost in quantitating oxygen because so much is taken up by the hemoglobin. We also do not as yet have measurement of the MMH remaining at any one time.

DR. SLONIM: I take it that no one has done any oxygen consumption with any of these types of hydrazines in the past?

MR. LEAHY: That was one point I was trying to determine from the literature. They seemed to be done in vessels which were loosely closed, and no specific amount of oxygen appeared to be available. So we went to complete oxygenation by having a humidified air supply blowing over a rapidly stirring mixture during reactions for one extreme and for the other extreme, having it totally or as totally reduced as possible, without using a chemical means of reducing our hemoglobin, and I have not gotten reproducible or dependable measurements in between the two extremes at the moment.

DR. DOST: If I might make a suggestion on methodology, if you could use a system in which there is no gas phase, that is to say, a solution of oxygenated hemoglobin, a solution of monomethyl, and mix them completely free of bubbles, so that there is no loss of oxygen to the atmosphere, no loss of MMH to the atmosphere where there is bound to be a certain amount of gas-phase reaction, (now I am doing some work with nitric oxide, which necessitates this kind of approach) and if you have completely oxygenated hemoglobin, of course, you can attain any dilution that you wish to use, and, of course, you have complete control of the amount of MMH which is going into the system. If you're lucky enough that the reaction with reference to MMH is complete, this is a great help in determining not only the molar ratios of heme and MMH reacted, but also the real influence of oxygen, whether it's in the gas-phase or attached as a ligand to hemoglobin.

MR. LEAHY: On that point it appeared the oxygen was consumed at approximately the same rate as the nitrogen we measured coming off, which was very similar to the air oxidation reaction that Vernot and others have reported here. I had hoped to get hold of a Gilson Oxygraph and do some measurements in the sealed system that way, but I was still debating on the effect of oxygen carrying capability of the blood and the effect of the shift, any shift in the curve with pH change with other changes that occurred, and it really gets quite involved trying to trace the oxygen down during this reaction. Methodology is the secret.